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May 2011

Vector-borne Infections

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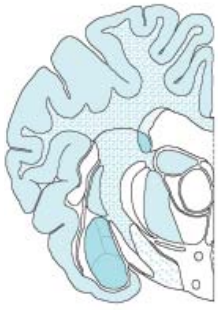

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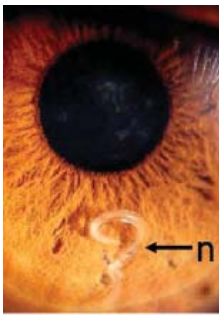

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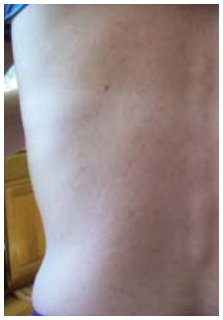

Another Dimension

Letters

About the Cover

Vector-borne Infections

Ronald Rosenberg and C. Ben Beard

Infections with vector-borne pathogens are a major source of emerging diseases. The ability of vectors to bridge spatial and ecologic gaps between animals and humans increases opportunities for emergence. Small adaptations of a pathogen to a vector can have profound effects on the rate of transmission to humans.

This issue of Emerging Infectious Diseases highlights the role of arthropod vectors in the origin and dissemination of emerging pathogens. As Woolhouse and Gaunt have pointed out (*1*), a substantial proportion of human pathogens are zoonotic and vector-borne, and they infect a substantial proportion of the world's population. Vector-borne pathogens also are prominent contributors to emerging disease. There are 3 principal reasons for this influence.

First, most major classes of pathogens have evolved agents that are capable of being transmitted by blood-feeding arthropods: viruses (e.g., yellow fever virus, Rift Valley fever virus), rickettsiae (*Rickettsia rickettsii*, *R. typhi*), bacteria (*Borrelia burgdorferi*, *Francisella tularensis*), protozoa (genera *Plasmodium* and *Leishmania*), and helminths (*Onchocerca volvulus*, *Wuchereria bancrofti*). Fungi seem to be the only category not represented.

Second, vectors bridge barriers that would prevent transmission by direct contact among humans and especially between animals and humans. These barriers are not only spatial but behavioral and ecological. Transmission of yellow fever virus between arboreal monkeys and humans by mosquitoes is the classic example, but there are many others; transmission of *B. burgdorferi*, the agent of Lyme disease, between evasive forest rodents and humans by ticks is just as exemplary. In such cases, direct contact between feral host and human would rarely take place. A corollary of this ability to bridge environments occurs when animals or humans move the pathogen from one vector-capable region to another. The introduction of West Nile virus into the United States in 1999 was a dramatic example, as was the recent introduction of Usutu virus to Europe from Africa in migrating birds (*2*). The potential for vector-borne zoonotic transmission to adapt to vector-borne human-to-human transmission is exemplified historically by dengue virus and *Plasmodium* spp., and more recently by Zika virus (*3*) and probably *P. knowlesi* (*4*).

Third, the complexity of vector transmission offers the pathogen increased opportunities to evolve. In almost no instances is the arthropod simply a vessel for transmission. Usually, the pathogen must move from the gut to the feeding apparatus to be transmitted. Mechanisms range from the relatively simple, as with the plague bacillus, *Yersinia pestis*, to the elaborately intricate, as with parasites in the genera *Plasmodium* and *Leishmania*. In these examples, the pathogen replicates in some fashion, which makes it dependent on an invertebrate host physiology much different from what it will encounter in its various vertebrate hosts. As a consequence, epidemic emergence can result from enhanced transmission independent of increased pathogenicity to humans. This is especially true of the arthropod-borne viruses (arboviruses) that infect humans, all of which are RNA viruses and have high potential mutability. A notable recent example is the chikungunya virus epidemic that swept through the Indian Ocean region beginning in 2006 and which is believed to have infected >2 million persons. A single-nucleotide polymorphism (SNP) in the virus genome accelerated its replication in the relatively common mosquito *Aedes albopictus*, usually a poorer host than *Ae. aegypti* mosquitoes (*5*). There is also evidence that an SNP enabled Venezuelan equine encephalitis virus to jump vectors, sparking the 1993 epidemic in Mexico (*6*), and it might have been an SNP in West Nile virus that increased its virulence to birds and influenced the shape of the epidemic in the United States

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(*7*). In none of these examples was increased pathogenicity to humans an apparent seminal factor in the epidemics.

Complexity of epidemiology and adaptive plasticity of pathogen and arthropod make the vector-borne diseases especially difficult to control, much less to eradicate. Vaccines are unavailable for all but a few diseases; and even when they are available, as for yellow fever, prevention can be difficult to achieve. The yellow fever epidemic that began in Uganda at the end of 2010 was the first in that country in 20 years. Tools for treatment are nearly as scarce. Falling behind in the race to keep up with developing resistance of *P. falciparum* to artemisinins is a specter that haunts malariologists, and treatment for visceral leishmaniasis remains too expensive and complicated to be widely practiced where it is most needed.

The constant development of pesticide resistance is even more worrisome than drug resistance because a pesticide can often be used to suppress vectors of many different pathogens. Even when pesticides are efficacious, their effectiveness is often compromised by human behavior and vector biology, as is often seen in campaigns against dengue. Changes in climate, land use, and transport will affect rates of pathogen emergence in ways we poorly understand. Fortunately, there is a growing appreciation by scientists and by funding agencies (*8*) that characterizing factors that influence pathogen and disease emergence are worthy goals for investigation, especially in those tropical environments where rapid change is most likely to incubate new pathogens.

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Intravenous Artesunate for Severe Malaria in Travelers, Europe

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Multicenter trials in Southeast Asia have shown better survival rates among patients with severe malaria, particularly those with high parasitemia levels, treated with intravenous (IV) artesunate than among those treated with quinine. In Europe, quinine is still the primary treatment for severe malaria. We conducted a retrospective analysis for 25 travelers with severe malaria who returned from malaria-endemic regions and were treated at 7 centers in Europe. All patients survived. Treatment with IV artesunate rapidly reduced parasitemia levels. In 6 patients at 5 treatment centers, a self-limiting episode of unexplained hemolysis occurred after reduction of parasitemia levels. Five patients required a blood transfusion. Patients with posttreatment hemolysis had received higher doses of IV artesunate than patients without hemolysis. IV artesunate was an effective alternative to quinine for treatment of malaria patients in Europe. Patients should be monitored for signs of hemolysis, especially after parasitologic cure.

Infection with *Plasmodium falciparum* malaria remains a major risk for European travelers returning from malaria-endemic areas. World Health Organization (WHO) guidelines recommend intravenous (IV) artesunate as first-line therapy for severe malaria (*1*). However, quinine is still the primary treatment for severe non–multidrug-resistant *P. falciparum* malaria in Europe (*2*) because IV artesunate is not registered for this indication, and the only commercially available product is not manufactured according to good manufacturing practice. Quinine has several adverse effects (e.g., cardiotoxicity, hypotension, hypoglycemia, and cinchonism), has a narrow therapeutic range, and must be administered 3×/d by rate-controlled infusion (*3*,*4*). In experienced hands, adverse effects can be minimized, but a major proportion of patients still experience moderate-to-severe side effects.

The efficacy and safety of artemisinins and their derivatives in oral, rectal, and intramuscular dosage forms have been widely studied (*5*–*11*). When administered intravenously, these drugs are useful for treatment of severe malaria because of their rapid parasite clearance, apparent absence of clinically relevant side effects, and simplicity of administration (e.g., by bolus injection). Since 1992, several studies in Asia (*5*,*6*,*8*–*10*) and a recent study of children in Africa (*11*) have shown better, or at least equivalent, survival rates for patients with severe malaria treated with artesunate than for those treated with quinine. This finding applies particularly to patients with severe malaria and hyperparasitemia (*10*).

Systematic data are not available for safety and efficacy of IV artesunate for treatment of severe *P. falciparum* malaria outside disease-endemic areas. In the United States, use of IV artesunate is monitored by the Centers for Disease Control and Prevention (Atlanta, GA, USA) under an investigational new drug protocol (*12*). In Europe, artesunate manufactured by the Guilin Pharmaceutical Factory No. 2 (Shanghai, People's Republic of China), which was used in all major trials of artesunate in Southeast Aia and Africa (*9*–*11*), is used. TropNetEurop (www.tropnet.net/about/contents/about_tropnet.html), a European surveillance network for tropical diseases, has been collecting data on artesunate use since 2005 (*13*).

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Severe malaria is rare outside disease-endemic regions. Thus, the limited numbers of patients in industrialized countries makes it difficult to conduct trials with sufficient statistical power to reproduce the survival benefit for IV artesunate observed in Southeast Asia (*10*). Nonetheless, these patients may benefit from the lower cardiotoxicity of artesunate than that of quinine and, because of more rapid parasite clearance, from reduction of time spent in intensive care units, in-hospital treatment, decreased use of exchange transfusion, and secondary complications. This finding is relevant for increased numbers of older persons who travel abroad to malaria-endemic areas, despite relevant cardiac or other medical conditions associated with a several-fold increased risk for complications and death caused by severe malaria (*14*). We report data for 25 patients with severe malaria who were treated with IV artesunate in 7 treatment centers in areas to which malaria was not endemic.

Study Characteristics

During January 2006–June 2010, we conducted a retrospective analysis of 25 patients from 7 treatment centers in Europe who were admitted to a hospital for *P. falciparum* malaria, which was classified as severe according to WHO criteria (*15*,*16*), and who received IV artesunate as the main antiparasitic therapy. The hyperparasitemia level for patients in a region to which malaria was not endemic was ≥5% (*15*). Patients treated at 7 centers, 4 in Germany (2 in Berlin, 1 in Heidelberg, and 1 in Düsseldorf), and 1 each in Denmark (Copenhagen), Sweden (Helsingborg), and Norway (Bergen), participated in the study. The Berlin (Charité University Medical Center), Heidelberg, Düsseldorf, Bergen, and Copenhagen centers are tertiary care academic teaching hospitals; the center in Helsingborg and the Armed Forces Hospital in Berlin are secondary care regional referral hospitals. The second Berlin center and the Bergen center provided data only for patients with posttreatment hemolysis; other centers provided data for all patients treated with IV artesunate. Anonymous treatment data were reported on case-reporting forms for severe malaria (TropNetEurop). The study was reviewed and approved by the ethics committee of the Charité Hospital in Berlin. Artesunate was obtained from the Guilin Pharmaceutical Factory No. 2 and stored at room temperature in all centers, according to the manufacturer's instructions.

Posttreatment Hemolysis

Serum and plasma of 3 patients with unusual posttreatment hemolysis in Berlin and Heidelberg (patients 6, 7, and 9) were tested for drug-induced autoantibodies, which react in the absence of the drug or its metabolites with erythrocytes, and for drug-dependent antibodies, which react only in the presence of the drug or its metabolites. Serum or plasma samples were available for testing from the time of artesunate treatment (patient 7), from the period of posttreatment hemolysis (patients 6, 7, and 9), or from the convalescent phase (7 and 16 months; patients 6 and 9).

Serologic testing was conducted by using standard gel card techniques (DiaMed, Cressier sur Morat, Switzerland). Artesunate was diluted in 0.9% NaCl at a concentration of 1.0 mg/mL. Ex vivo antigens (urine) were obtained from 2 patients receiving IV artesunate to detect reactivity to artesunate metabolites. Serum samples were tested for reactivity with artesunate solution or urine metabolites by using the indirect antiglobulin test and a drug-dependent–antibody test with the gel card technique (*17*–*19*). Cumulative doses and treatment duration (days) were compared between adult patients with and without signs of posttreatment hemolysis by using the Mann-Whitney U test.

Patient Characteristics

One child and 24 adults (mean ± SD age 44.1 ± 16.1 years; 14 male and 11 female patients) treated with IV artesunate for severe malaria during January 2006–June 2010 were included in the study (online Appendix Table, www.cdc.gov/EID/content/17/5/771-appT.htm). Eighteen patients were travelers from Europe to malaria-endemic areas, and 7 patients were immigrants who returned from malaria-endemic countries after having visited friends and relatives. With the exception of patient 13, who was a short-term visitor to Germany from Chad, all other patients who visited friends and relatives had permanently left their home countries for >5 years before becoming infected.

Hyperparasitemia (range 5%–51% parasitized erythrocytes) in 20 (80%) patients and cerebral malaria in 8 (32%) patients were the most common severe malaria-defining criteria observed. Seven patients (28%) had renal failure, and 2 (8%) required hemodialysis. Respiratory failure caused by severe shock developed in 1 patient; this patient required therapy with vasopressors and mechanical ventilation for 6 days. Repeated chest radiographs did not show pulmonary edema or pneumonia. Shock developed in 4 patients (patients 3, 4, 13, and 25); these patients required vasopressor therapy.

Antimalarial Therapy

Details on dosage, treatment duration, and concomitant therapy are shown in the online Appendix Table. All but 3 patients received IV artesunate as first-line therapy. Therapy for patient 1 was changed to IV artesunate after complications (bradycardia) caused by the first dose of quinine. Therapy for patients 10 and 13 was 1 dose of artemether/lumefantrin or IV quinine, respectively, before transfer to a treatment center to avoid a delay in treatment initiation.

Patients 3–8, 15, 16, 18, and 19 received the dosing regimen for artesunate initially recommended by WHO (*16*): after an initial dose of 2.4 mg/kg, therapy was continued with 1.2 mg/kg every 12 hours and then 1.2 mg/kg every 24 hours. Patients 9–13 and 19–25 received artesunate, 2.4 mg/kg/dose. Therapy for all but 6 patients was changed to oral artemether/lumefantrine or atovaquone/proguanil after rapid clinical improvement and ability to swallow on days 3–4 of treatment. Different batches of artesunate were used in the Berlin and Heidelberg treatment centers. Batch information was not available from centers in Helsingborg, Copenhagen, and Bergen and the second center in Berlin. Six patients in whom posttreatment hemolysis occurred were treated for 4 years.

Efficacy

In all patients with hyperparasitemia, parasite load was reduced ≈1 $\log_{10}$ after 24–36 hours. All but 1 patient were free of parasites 36 hours–134 hours after the initial dose of artesunate. Parasite clearance was delayed (158 hours) in 1 patient (patient 7). In this patient, infection with HIV was diagnosed (CD4 count 382 cells/μL). Mean ± SD parasite clearance time was 81.2 ± 35.4 hours for all patients treated with IV artesunate as first-line drug, who had an initial parasitemia levels >1% and for whom data were available (patients 2–12, 14, and 20–24), and 78.9 ± 29.5 hours for patients not infected with HIV.

Tolerability

IV artesunate was generally well tolerated; there was no evidence of hemodynamic, cardiac, or allergic adverse reactions. Six patients from 5 treatment centers showed unusual hemolytic anemia, which recurred after clearance of parasites and was diagnosed 14–31 days after the first dose of IV artesunate (patients 6, 11, and 23) or persisted after the end of treatment until the end of the fourth week after the first dose of IV artesunate (patients 7, 9, and 25). Laboratory findings and typical patterns of hemolysis are shown in the Table and the Figure.

Patient 6 was treated with artesunate and doxycycline; she had malaria-related hemolysis and an initial hemoglobin level of 11.3 g/dL. This patient was discharged in good clinical condition on day 10 (hemoglobin level 7.7 g/dL, which had been stable for the past 4 days) and had a decreased lactate dehydrogenase (LDH) level (317 U/L). On day 15, this patient was readmitted because of severe anemia caused by recurring hemolysis (hemoglobin 5.7 g/dL, LDH 1,437 U/L). Glucose-6-phosphate dehydrogenase (G6PD) deficiency and antibody-mediated hemolysis were excluded as causes (negative result for Coombs test). The reticulocyte count was high (10.2%). After receiving 2 units of packed erythrocytes, the hemoglobin level of this patient remained stable.

Patient 11, who was treated in Helsingborg, received IV artesunate for 7 days. On day 15, laboratory parameters were indicative of secondary hemolysis. The reticulocyte count was within reference limits initially and was near the upper reference value during secondary hemolysis. Patient 23, who was treated in Bergen, had a similar episode of recurring and intense hemolysis after 4 days of treatment with IV artesunate, beginning on day 15, which required readmission to the center and blood transfusion. Results of the Coombs test were repeatedly negative, G6PD deficiency was ruled out, and reticulocytes values were 2.3× the upper reference value.

Other patients showed patterns of persisting hemolysis. Patient 7 was discharged from the hospital in Berlin 14 days after the first dose of IV artesunate (treatment duration 7 days) with a hemoglobin level of 8.2 g/dL, which was stable for 10 days. On day 32, this patient was readmitted to the University Hospital in Heidelberg with a hemoglobin level of 6.1 g/dL and signs of hemolysis (LDH 805 U/L). The patient received 2 units of packed erythrocytes and was discharged 3 days later in good clinical condition. Hemolytic activity decreased over the next 10 days.

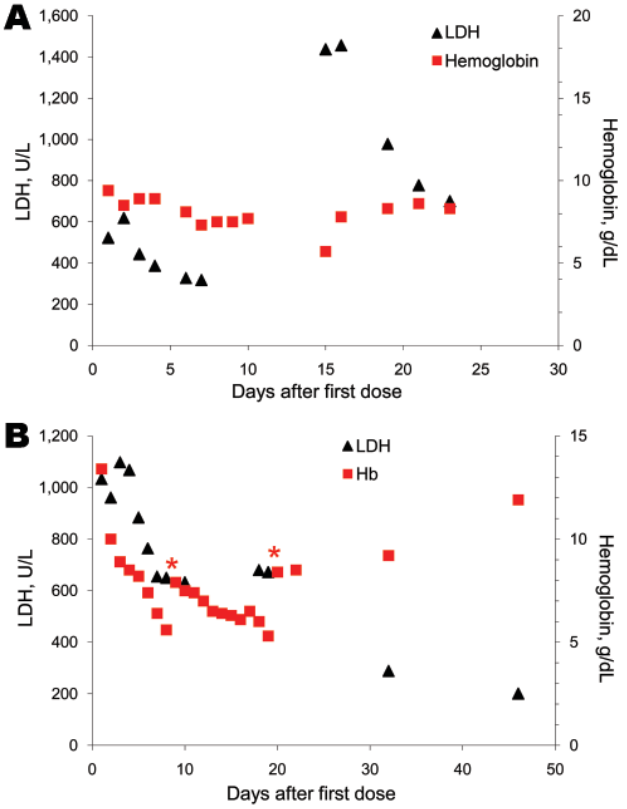

Figure. Typical patterns of hemolysis in 2 travelers with severe malaria treated with intravenous artesunate, Europe, January 2006–June 2010. A) Patient 6 with recurring hemolysis. B) Patient 9 with persisting hemolysis. LDH, lactate dehydrogenase. * indicates blood transfusion. Gaps between symbols indicate periods when samples were not obtained.

Table. Laboratory test results for 6 patients with posttreatment hemolysis who had been treated with intravenous artesunate for severe malaria, Europe, January 2006–June 2010*

Patient no.	Initial parasitemia level, %	Levels at first examination		Treatment duration, d	Parasite clearance, d	Levels at end of treatment		Day of diagnosis of hemolysis†	Levels at diagnosis of hemolysis		Other test results
		Hb, g/dL	LDH, U/L			Hb, g/dL	LDH, U/L		Hb, g/dL	LDH, U/L	
6	30	11.3	765	7	4	7.7	317	15	5.7	1,437	Coombs negative, reticulocytes 10.2%, G6PD deficiency ruled out
7	20	13.2	1,359	7	7	8.2	NA	32‡	6.1	805	None
9	30	13.4	1,033	4	5	7.6	650	19‡	5.3	672	None
11	4	13.4	904	7	2	9.8	311	15	7.8	660	Standard reticulocyte count
23	9	15.5	490	4	2	11.1	571	15	5.7	1,489	Reticulocytes >2× upper reference value, haptoglobin <0.1g/L, Coombs negative
25	10	14.2	570	3	NA	7.8	454	16‡	5.8	444	Reticulocytes 3× upper reference value, haptoglobin <0.08 g/L (day 14), G6PD deficiency ruled out

*Hb, hemoglobin; LDH, lactate dehydrogenase; G6PD, gluose-6-phosphate dehydrogenase; NA, not available.
†After first dose of artesunate.
‡Patients had persistent hemolytic activity after the end of malaria treatment.

Patient 9 was treated in Heidelberg and received IV artesunate for 3 days, followed by oral artemether/lumefantrine for another 3 days, and was parasite free after 96 hours. Intense and persisting hemolysis resulted in a hemoglobin level of 5.6 g/dL on day 8 (LDH 633 U/L). This patient received 2 units of packed erythrocytes, and hemoglobin level increased to 7.9 g/dL. On day 19, hemoglobin level decreased to 5.3 g/dL because of persistent hemolysis (LDH 672 U/L); he was again given transfusions of packed erythrocytes. Hemolytic activity decreased after day 22, and LDH levels returned to reference values on day 46. Patient 25 showed a similar pattern of persistent hemolysis, which gradually decreased after day 21, after malaria therapy.

In multiple repeat thin blood films used to determine parasitemia levels, no abnormalities in erythrocyte morphology were observed. To identify causes of posttreatment hemolysis, cumulative doses of IV artesunate and treatment durations were compared among all adult patients. Patients with posttreatment hemolysis had received higher doses of IV artesunate than patients without observed hemolysis (mean ± SD cumulative dose 12.8 ± 3.3 mg/kg vs. 7.6 ± 2.9 mg/kg; $p = 0.006$ in all adult patients) and were treated for longer periods (mean ± SD 5.8 ± 1.6 days vs. 3.6 ± 1.7 days; $p = 0.038$).

Immunohematologic Tests

Free indirect antibodies against globulin were not detected in serum or plasma from 3 patients (patients 6, 7, and 9) at the Berlin and Heidelberg treatment centers who had prolonged posttreatment hemolysis. Presence of drug-dependent antibodies in serum or plasma of patients was investigated by using as test substrates an artesunate solution and urine (artesunate metabolites) of patients receiving artesunate therapy; antibodies were not detected.

Clinical Outcome

All patients survived and all complications related to severe malaria resolved at time of hospital discharge for all but 1 patient. Patient 2 had a more severe clinical course (respiratory and renal failure), and required further rehabilitation and physiotherapy because of critical illness (neuropathy) that developed while he received prolonged intensive care and immobilization. Unusual hemolysis in 6 patients resolved spontaneously during weeks 3–6 after the first dose of IV artesunate.

Conclusions

Data from large multicenter trials on use of parenteral artesunate are limited to malaria-endemic regions, particularly Southeast Asia. We report data on use of parenteral artesunate for patients with severe malaria outside malaria-endemic areas, who were treated according to intensive care standards in Europe. In these patients, treatment with IV artesunate was effective and induced rapid parasite clearance. The only other report of a series of patients treated with IV artesunate for severe malaria outside malaria-endemic areas was from Norway; outcomes for 9 patients were good, and adverse reactions related to IV artesunate were not observed (*21*).

Parasitemia levels took longer to clear for participants in our study than those in a study in Thailand (*9*) (mean ± SD 81.2 ± 35.4 hours vs. 62.5 hours, 95% confidence interval 53.4–71.8 hours). In contrast with uncomplicated malaria, parasite clearance times for severe malaria are difficult to compare when different drug regimens (concomitant and sequence therapy) have been used. In addition, all patients in our study were considered nonimmune.

High parasitemia levels in severe malaria are more likely to develop in nonimmune patients; such patients are more likely to receive exchange transfusions. Physicians treating patients in our study decided not to use exchange transfusions because they have unproven benefits. Artesunate has been shown to be particularly effective in reducing mortality rates among patients with parasitemia levels >10% (*10*). Therefore, patients from Europe may benefit more from treatment with artesunate than with quinine.

Unusual episodes of hemolysis developed in 6 patients in our study. These patients had clinical signs caused by anemia during the third week of treatment with IV artesunate or had persistent signs of hemolytic activity until 6 weeks after the first dose of IV artesunate. In all cases, physicians in different treatment centers were unaware of other cases at that time, and hemolysis was not immediately considered to be induced by IV artesunate. Thus, a follow-up of patient serum samples and pharmacologic analysis of drugs used was not conducted. An additional case of Coombs-negative, posttreatment hemolysis in a European traveler during the third week after receiving IV artesunate/quinine therapy in Tanzania was reported from the Netherlands (R.M. Peerenboom, unpub. data). Despite these observations, it is not appropriate to infer incidence rates from our study regarding the incidence of posttreatment hemolysis in patients treated with IV artesunate because in 2 centers not all patients were available for inclusion. Known and possible causes of hemolytic anemia in association with malaria or antiparasitic therapy include blackwater fever, artemisinin-induced reticulocytopenia, direct hemolytic effects of the drug, and drug-induced immune hemolytic anemia.

G6PD deficiency is the basis for primaquine-, quinine- (*22*), and tafenoquine- (*23*) induced hemolytic anemia, but it was not identified in patients tested in our study. Blackwater fever, which causes acute hemolysis and hemoglobinuria in the early course of malaria treatment, was observed in the South East Asian Quinine Artesunate Trial (*10*) for quinine and artesunate (5% vs. 7%). Late onset, prolonged duration, and recurrence are not typical for blackwater fever as the cause of hemolysis in our patients. A temporary depression of reticulocytopoesis 3–7 days after the first dose of artemisinin derivatives has been reported in other studies (*5*,*24*,*25*). This phenomenon was not found for patients in our study for whom reticulocyte levels were determined.

IV artesunate is rapidly hydrolyzed to the active metabolite dihydroartemisinin. Because dihydroartemisinin has a short half-life (*26*), prolonged hemolysis after stopping treatment with IV artesunate and the recurring hemolysis in 2 patients suggest that a direct hemolytic effect of the drug is unlikely, although contaminants may have a longer half-life. Drug-induced immune hemolytic anemia, which involves production of drug-induced, irregular autoantibodies against erythrocytes, is typically associated with administration of different drugs (e.g., cephalosporins, quinine, penicillin, diclofenac, or rifampin) (*19*,*27*,*28*).

Formation of drug-dependent immunoglobulin (Ig) G or IgM leads to hemolysis and complement activation only in the presence of the causative drug; other drug-independent IgG types can cause hemolytic anemia in the absence of the drug (*29*). This second type of hemolysis occurs without complement activation and, in most cases, has a less severe clinical course. This unusual pattern of hemolysis was not observed in large clinical trials conducted with IV artesunate obtained from the same manufacturer in China (*9*,*10*) or in studies of oral artesunate. One case of a similar phenomenon was reported in Japan (*30*). However, in previous trials, patients were not routinely followed up, and cases of prolonged or recurring hemolysis might have been missed. Results of immunohematologic tests in our study did not indicate drug-induced or drug-dependent hemolysis. Because these tests had to be performed with frozen blood samples, results may have been influenced by the quality of the samples. However, the consistently negative Coombs test result for fresh blood from 2 of our patients with hemolysis suggests that hemolysis induced by autoimmune mechanisms is unlikely.

Patients with posttreatment hemolysis had received a higher cumulative dose of IV artesunate and were treated for longer periods. This observation supports the hypothesis that hemolysis might occur as a consequence of artesunate treatment in a dose-dependent manner. However, because this study did not have a prospective design and patients were not routinely followed up for signs of hemolysis in all centers, we cannot exclude undetected cases of hemolysis in our study.

The underlying cause of posttreatment hemolysis in our study of travelers is still unknown. Because IV artesunate currently produced in China is not manufactured according to standards of good manufacturing practice used in Europe, contaminants might have caused direct or antibody-mediated hemolysis in our patients. However, the manufacturer of IV artesunate recently passed the WHO drug prequalification program (*31*). Hemolysis in 5 centers in Europe over a period of 4 years suggests that contamination in a batch of IV artesunate is unlikely. Other reported artesunate-related adverse reactions, such

as hypersensitivity reactions (*32*) and vestibulocochlear disturbances (*33*,*34*), were not observed in our study.

The role of IV artesunate for treatment of severe malaria in patients treated in Europe remains to be defined. However, it should be considered for patients with hyperparasitemia, patients with medical conditions limiting or prohibiting use of quinine, or patients in whom quinine-related adverse reactions are observed. Efficacy and safety profiles of IV artesunate should be prospectively evaluated, and patients should be monitored for signs of hemolysis after parasitologic cure. Reducing the cumulative dose of IV artesunate by early initiation of oral treatment might help reduce risk for posttreatment hemolysis. Improving availability of IV artesunate produced according to standards of good manufacturing practice used in Europe or the United States would constitute a major step in improving therapy for severe malaria.

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Lessons Learned about Pneumonic Plague Diagnosis from 2 Outbreaks, Democratic Republic of the Congo

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Learning Objectives

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

- Describe transmission, presentation, and course of pneumonic plague
- Describe how outbreaks of pneumonic plague in the Democratic Republic of the Congo were recognized
- Describe frontline response strategy to pneumonic plague that will facilitate implementation of the first control measures

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Pneumonic plague is a highly transmissible infectious disease for which fatality rates can be high if untreated; it is considered extremely lethal. Without prompt diagnosis and treatment, disease management can be problematic. In the Democratic Republic of the Congo, 2 outbreaks of pneumonic plague occurred during 2005 and 2006. In 2005, because of limitations in laboratory capabilities, etiology was confirmed only through retrospective serologic studies. This prompted modifications in diagnostic strategies, resulting in isolation of *Yersinia pestis* during the second outbreak. Results from these outbreaks demonstrate the utility of a rapid diagnostic test detecting F1 antigen for initial diagnosis and public health management, as well as the need for specialized sampling kits and trained personnel for quality specimen collection and appropriate specimen handling and preservation for plague confirmation and *Y. pestis* isolation. Efficient frontline management and a streamlined diagnostic strategy are essential for confirming plague, especially in remote areas.

Plague is a zoonotic disease caused by the bacterium *Yersinia pestis,* an agent circulating among small mammals and fleas (*1*–*3*). Human infection is usually transmitted by the bite of an infected flea, and bubonic plague is the most common form of the disease. The

illness can progress into advanced clinical forms of septicemic and pneumonic plague. Pneumonic plague is of serious concern because of the potential for human-to-human transmission from aerosolized bacteria spread through coughing. Pneumonic plague can lead to localized outbreaks, or even devastating epidemics, because the infectious dose by inhalation can be as low as 100–500 organisms (*4*). Untreated, pneumonic plague usually leads to death within 2–4 days after respiratory exposure; in some instances, death occurs as rapidly as 24 hours after exposure (*1*,*3*,*5*,*6*). The rapid onset and high lethality are its only distinguishable clinical features as the disease otherwise manifests itself as a severe respiratory infection that could be caused by various pathogens.

In regions where clinicians are unfamiliar with plague, risk of misdiagnosis is high, and specific diagnostic tools are often not readily accessible in remote areas. Identification of the causal agent is critical for implementing immediate public health measures in the community. Furthermore, in previously plague-free areas, confirmation of the diagnosis may lead to the identification of the emergence of a new natural focus that requires a revised public health strategy.

Rapid diagnostic tests (RDTs) are available and can be effective for helping manage such situations; however, they do not replace bacterial isolation, which remains the most accurate method and enables crucial antimicrobial drug susceptibility testing. In the absence of bacterial isolation, plague confirmation requires serologic detection of a 4-fold rise in plague-specific antibodies, or, in plague-endemic regions, a positive RDT (*7*). Whichever technique is used, plague confirmation depends on appropriate sample collection, effective sample preservation measures, and direct transportation of samples to diagnostic laboratory facilities, as illustrated in the 2 pneumonic plague outbreaks we discuss. We highlight important lessons on frontline sample collection methods and transport and provide an awareness of the challenges faced in regard to diagnostic strategies in remote, war-torn regions of the world.

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The Outbreaks

Plague remains a concern in several countries, particularly in Africa (*8*–*12*). The Democratic Republic of the Congo (DRC), a war-torn country, has the most active focus of plague worldwide. In the northeastern region of Ituri, >1,000 suspected cases are reported each year (*7*,*12*,*13*). In January 2005, an outbreak of highly lethal pneumonia occurred in a diamond mining camp in a remote area of the Oriental Province (Figure 1), 25 km from the village of Zobia, Bas-Uele (*13*,*14*). Clinical signs and fast spread of the disease lead to the suspicion of pneumonic plague. No previous cases of plague had been reported in this region, which has a history of severe security problems and where at the time of the outbreak a United Nations peacekeeping operation was ongoing. In August 2006, an outbreak of a similar nature occurred in a gold mining camp 200 km from the Zobia camp, near Bolebole, Haut Uele (*15*).

Investigative Plans

At the request of Congolese officials, World Health Organization (WHO) response teams intervened in both outbreaks. However, because of a delayed alert and bureaucratic challenges, the teams arrived 2 months after onset of the epidemics, which limited the number of case-patients who were available for clinical examination and appropriate sampling. As a result of the circumstances, the following investigative plan was established.

The suspected plague deaths that occurred before the WHO response team arrival were attributed to plague on the basis of the epidemiologic context, the reported clinical signs, and the rapid disease onset typically observed for pneumonic plague. However, no postmortem specimens could be taken for confirmation. Suspected convalescents were tracked, interviewed, and blood samples were collected in standard serologic tubes by a clinician and stored at 4°C for retrospective serologic testing.

Each new patient with clinically suspected pneumonic plague was admitted to an isolation center to receive the first doses of the national protocol treatment. Blood was immediately sampled and sputum samples were collected by a trained biologist from the National Plague Reference Laboratory who supported the WHO mission. Sampling kits, designed by the Institut Pasteur de Madagascar (IPM; Antananarivo, Madagascar) for improving laboratory confirmation of plague cases in this country, were used to collect and prepare sputum samples for transport. The kits contained the necessary equipment for sampling either sputum or bubo aspirate and provided an effective method for collecting pure samples with minimal chance of contamination. One kit was required per patient; each

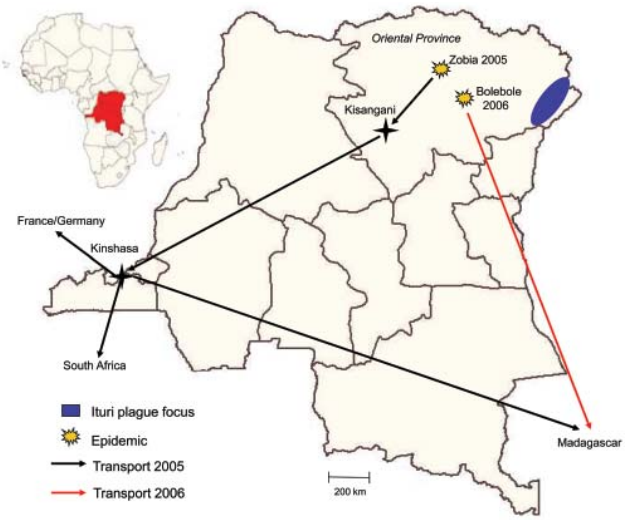

Figure 1. Transport routes of clinical specimens from 2 pneumonic plague outbreaks, Democratic Republic of the Congo, 2005 and 2006.

contained a sterile syringe for sample extraction from a bubo or sputum sampling cup, an Eppendorf tube containing 1 mL of phosphate-buffered saline (PBS) for sample dilution, a culture tube with Cary Blair (CB) medium, a sterile swab for placement of pure sample into CB media for transport, a sterile test tube for RDT, isopropyl alcohol and antiseptic towelettes, and a patient record form. Safety procedures included use of proper personal protective equipment and appropriate waste management.

Specimen Handling and Transportation

In the 2005 Zobia outbreak, handling and transportation of the specimens were planned as follows. Sputum specimens were immediately evaluated on site by a biologist of the National Plague Reference Laboratory using direct microscopy and RDT. The specimens were then transported to a temporary second-line regional laboratory in Kisangani, DRC, by air (2 hours) on roughly a weekly basis. In Kisangani, a biologist from IPM who was assigned to the WHO mission performed culture identification for *Y. pestis* and direct microscopy and prepared specimens for the twice weekly air shipment (>1,000 km) to the Institut National pour la Recherche Biologique in Kinshasa, DRC. At the time of arrival at Institut National pour la Recherche Biologique, specimens were further distributed by commercial air carriers to IPM in Antananarivo, Madagascar; the National Institute for Communicable Diseases (NICD) in Johannesburg, South Africa; and the Centre de Recherche du Service de Santé des Armées in Grenoble, France, for additional analyses and serologic testing (Figure 2, panel A).

In the 2006 Bolebole outbreak, a streamlined strategy was defined to limit sample passages and delays in transport. Emphasis was placed on RDT and direct microscopy on site, but no other manipulation of samples was allowed. Regional and national steps were bypassed and samples were sent directly to the international reference laboratory at IPM, where confirmatory tests were performed (Figure 2, panel B).

Specimen Analyses

Sputum smears were stained (Gram and Wayson) and directly examined by microscopy according to standard protocols (*16*). In addition, sputum was extracted from swabs stored in CB by using 1 mL of PBS and cultured by using standard methods for plague isolation as recommended by WHO (*16*). Suspect colonies were confirmed by using standard biochemical tests and phage lysis as described (*16*–*19*). The above methods were performed at Kisangani, NICD, and IPM during the first outbreak and only at IPM during the second outbreak.

RDT for *Y. pestis* specific F1-antigen detection by immunochromatography was performed at the frontline. The dipstick is contained in an individually vacuum-sealed package with desiccant to maintain stability and sterility and is used with the sampling kit described above. The specimen was processed by using 0.5 mL of thick sputum diluted in 1 mL of PBS and homogenized by using a sterile syringe. A 200-μL aliquot of homogenate was placed in a sterile test tube and the dipstick applied. The test was considered positive when 2 pink lines (control line + test line) appeared after 15 minutes as described (*18*). To simplify the procedures in the field, semiquantitative grading was not requested.

One serum sample was drawn as early as possible after onset of symptoms and a second serum sample 10 days later to allow for the development of an antibody response. A positive result was determined by a 4-fold increase in the antibody titer from the first to the second sample (*1*). Serum was transported at 4°C or at ambient temperature and filter-sterilized upon arrival. ELISA detecting anti-F1 immunoglobulin (Ig) G was performed as previously described (*20*) at Centre de Recherche du Service de Santé des Armées.

Direct fluorescent antibody (DFA) staining was performed at NICD on sputum specimens from the first outbreak according to standard protocols (*16*,*21*,*22*). Smears were a thin impression (touch preparation) of sputum on swabs transported in CB medium. The presence of plague bacilli was tested through the direct binding of fluorescein isothiocyanate–conjugated F1 antibody to antigen. A smear was positive when bright, intense green staining around bacteria was observed.

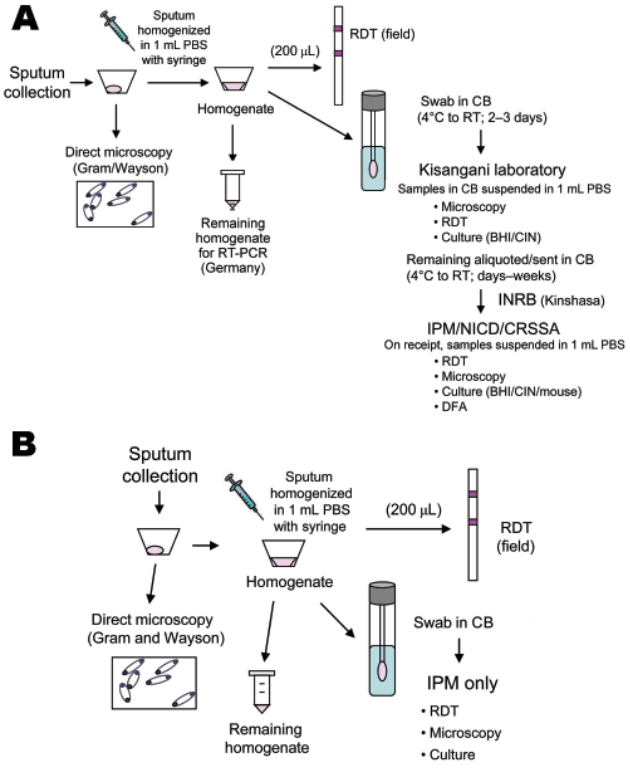

Figure 2. Flow of sample processing for specimens for pneumonic plague outbreaks in Zobia, Democratic Republic of the Congo (DRC), 2005 (A), and Bolebole, DRC, 2006 (B). PBS, phosphate-buffered saline; RDT, rapid diagnostic test; RT, reverse transcription; CB, Cary Blair; BHI/CIN, brain–heart infusion; cefsulodin-Irgasan-novobiocin; INRB, Institut National pour la Recherche Biologique; IPM/NICD/CRSSA, Institut Pasteur de Madagascar/National Institute for Communicable Diseases/Centre de Recherche du Service de Santé des Armées; DFA, direct fluorescent antibody

Field Implementation

The first plague outbreak (Zobia) took place between December 15, 2004, and March 11, 2005, and resulted in 130 cases, all pneumonic except for 2 septicemic, and 57 deaths (44% fatality rate). Forty-eight deaths had already occurred. No specimens had been taken when the WHO response team arrived in the field on February 19. At the time of arrival, specimens were collected from 46 patients with acute illness (2 had a pair of blood samples collected), and from 41 convalescents (4 had a pair of blood samples collected) (Table). *Y. pestis* was not isolated from any specimen from a patient with acute illness, but samples from 5 of the 6 patients with paired blood samples converted. Of note, 18 of 37 (49%) of sputum specimens from patients with acute illness tested by RDT were positive.

The second outbreak (Bolebole) lasted from August 21 through October 21, 2006. In a preliminary investigation, 15 sputum samples were collected. However, these samples were stored and transported in dry containers and did not yield results indicative of plague when they were analyzed in a reference laboratory. A second investigation in cooperation with the National Plague Reference Laboratory suspected 5 other patients were infected. Forty-five deaths were reported. RDTs were performed in the field and 2 immediately showed positive results, prompting a new request for an international intervention, which led to the third investigation. During this phase, specimens were collected from 98 patients having acute illness (of which 2 specimens were excluded) and 19 convalescents; no paired sera were collected (Table). *Y. pestis* was isolated and confirmed by its biochemical profile from 4 sputum specimens. Of note, 23 of 96 (24%), including all specimens in which *Y. pestis* was isolated, were positive by RDT. Local authorities first reported 1,597 suspected pneumonic cases and 54 deaths. However, the international mission observed a significant overreporting caused by inappropriate case definition. Most of the medical files were not able to be retrieved, but the number of cases was estimated at 162 on the basis of information included in the registries. We can retrospectively describe the 2 outbreaks according to the WHO case definition as follows. In Zobia, in the absence of the isolation of *Y. pestis*, 5 cases were confirmed (seroconversion), 10 were probable (≥2 positive tests), and 115 remained suspect. In Bolebole, 23 cases were confirmed (4 isolations plus 19 positive RDT), 22 were probable (direct examination or single serology positive), and 117 remained suspect.

Specimen Handling and Transport

Pneumonic plague was suspected late after the onset of the outbreak in Zobia. No clinical specimen had been taken before the arrival of the WHO response team because first-line staff had not been properly trained and appropriate sampling materials were not available. After deployment, and under direct management of the response team, the frontline laboratory conducted RDT and direct microscopy; results indicated plague. All patients with suspected plague were subsequently isolated and treated, and close contacts received chemoprophylaxis as recommended by WHO (*1*). During this outbreak, difficulties with isolating *Y. pestis* were anticipated and a second-line laboratory in Kisangani was established. Unfortunately, the functions of this laboratory were limited by power and water shortages, which restricted the capacity to perform. As such, this laboratory in practice served only as a logistic platform for the air shipment of specimens to Kinshasa. Shipments from Zobia to Madagascar took 8–40 days (median 18.5 days). Sputum samples in CB were stored at 28°C–30°C, and serum was stored at 4°C.

At Bolebole, greater emphasis was placed on front-line sampling, avoiding dilutions, and multiple handling of specimens. Only direct microbiology and RDT for

Table. Laboratory results for 87 and 117 suspected cases of pneumonic plague during outbreaks in 2005 and 2006, respectively, Democratic Republic of Congo*

Location, year	No. cases for which samples were collected from patients	No. positive samples/no. tested (% positive)					
		RDT	Microscopy	*Yersinia pestis* isolation	DFA	PCR	IgG
Zobia, 2005†	Acute, 44	17/35 (48.6)	31/44 (70.4)	0/44	6/43 (13.9)	2/42 (4.8)	7/18 (3.9)
	Paired samples, 6	1/2 (50)	1/2 (50)	NA	0/2	0/2	6//12 (50.0)
	Convalescent, 37	NA	NA	NA	NA	NA	12/37 (3.2)
Bole, 2006‡	Acute, 98	23/96 (24.0)	38/67 (56.7)	4/96 (4.2)	NA	NA	2/89 (2.2)
	Convalescent, 19	NA	NA	NA	NA	NA	0/19 (0)

*RDT, rapid diagnostic test; DFA, direct fluorescent antibody test; PCR, real-time PCR; Ig, immunoglobulin; NA, not applicable.
†Of 130 cases identified, samples were collected from 87 patients.
‡Of 162 cases identified, samples were collected from 117 patients.

F1 detection were conducted on site, which provided results indicating plague. Samples were then obtained by specimen collection kits and sent in good condition. Samples were stored at the same temperatures as during the first outbreak, were transported once a week by road from Bolebole to Isiro (2 days), and then shipped by air to Kinshasa on an 8-hour flight with 3 intermediate landings. Finally, specimens were shipped to Madagascar weekly by commercial airlines. In total, the transport of samples from Bolebole to IPM took 4–5 days.

Use of RDT

Cost-effective, easy to use, and reliable, the RDT can be considered as an alert tool. It has proven to be rapid and easy to use at the bedside with 100% specificity and sensitivity (*18*). Importantly, detection of the F1-antigen is possible even in cases that have been previously treated, which was common for many of the patients in the outbreaks we describe. However, the RDT is not a screening test and must be used only to confirm a clinical suspicion. The RDT is also helpful for outbreak control. In both Zobia and Bolebole, one of the most challenging and time-consuming activities was managing the close contacts (≈25 for each suspected case). Even though the RDT results have no confirmatory value during the early phase of the outbreak, they enable management of close contacts to be focused around cases defined as probable within 15 minutes. Notably, the RDT becomes a field confirmation test after *Y. pestis* has been identified on site (*7*).

Specimen Collection and Testing

In addition to sputum samples, a serum sample was drawn from all patients admitted to the isolation center as well as from convalescents. However, in circumstances as described above, it was challenging to obtain a second blood sample from the patients 10 days later. As for the suspect cases that occurred before the arrival of the response team, motivation for testing was weak and no suspect patients returned. This was because most patients were young male adults, who returned to mining activities after cure and release from the isolation center. Any kind of control was perceived only as a limitation to business as usual. Overall, only 6 pairs of serum samples were obtained from patients treated at an early stage in Zobia, despite a strong emphasis being placed on serology for confirmation. The situation was worse in Bolebole, as no paired serum samples were obtained.

Isolation of *Y. pestis* from sputum was desired but challenging because of administration of antimicrobial drugs to patients before specimen collection, the nonsterile nature of the specimen, the slow growth of *Y. pestis* in culture, and prolonged specimen handling and transport. In addition, results from DFA and real-time PCR testing may also have been affected by prolonged handling and transport.

Because of the limitations in the field, it is difficult to extract information in regard to the respective performances of different diagnostic techniques. RDT is an established test for Africa and is known for its high sensitivity and specificity. If nearly half of the tested patients were positive in Zobia and 24% in Bolebole (Table), this can be due to inappropriate specimens (saliva instead of sputum) or a misdiagnosis. However it should be noted that 4 of 5 patients with confirmed plague in Zobia had a positive RDT result (DFA analyses were positive for 2 of them). In Bolebole, the 4 patients with plague confirmed by culture also had a positive RDT result.

Scope of the Outbreak

The primary objective of the laboratory investigation was to confirm the existence of ongoing pneumonic plague in the communities, to set up an ad hoc control strategy, and to stop the spread of disease. With regard to the magnitude of the 2 outbreaks, one cannot discard the possibility that a certain number of reported cases were not due to plague. However, one cannot discard the possibility that several plague cases were neither diagnosed nor reported. This is particularly true for the period that preceded the international intervention. In Zobia, to be as specific as possible, only the patients who died of a short-course and severe febrile pneumonic syndrome at this period were considered to have died of the plague. But what was the

number of survivors? This is difficult to project. The case-fatality ratio observed during the mission (11.0%) cannot be applied at the preintervention period because case management had greatly improved. In addition, the peak of the outbreak had passed, and the number of true plague cases among those clinically detected was probably lower because less than half of the patients with acute illness had a positive RDT (Table). The situation was similar at Bolebole.

Strategy for Sub-Saharan Africa

Rural Africa reports >95% of all plague cases worldwide. The cases usually occur in areas where the disease is endemic, but emergence is not exceptional. In plague-endemic endemic areas, priority must be given to reinforcement of the local diagnostic capacities. Sustainable and simple operating procedures should be promoted, and specimens should be treated locally or regionally as much as possible, avoiding challenging transport issues. Specimen collection kits and RDT must be provided for first-line detection and collection. Promoting RDT use, however, is much more than prepositioning dipsticks. RDT use must include training, supervision, and the necessary materials to appropriately collect and process the samples. Efforts must be given priority in districts with endemic plague and neighboring ones. Therefore, RDT should be part of a global strategy to reinforce the surveillance and control capacities, including laboratory diagnosis, at the district or provincial level. As shown in DRC, a provincial-level laboratory can perform biochemical identification from culture, confirm RDT results, and supervise RDT use in the framework of a plague surveillance and control strategy.

In previously plague-free areas, the approach is different. Any emergence is considered a public health emergency of international concern to be reported to WHO. Such situation requires a national or international response, which must include professionally trained and equipped teams to appropriately manage the outbreak and direct connections to an international reference laboratory. Specimen collection kits and RDT are efficient and easy-to-use tools for the investigation and the first control measures. However, the absolute need to demonstrate the etiology of the outbreak justifies any additional efforts that human and technical resources specifically deployed allow. Even if only a seroconversion is confirmatory, a unique seropositivity is considered an interesting finding in an area where *Y. pestis* is presumed not to circulate. The practical difficulties experienced in Zobia and Bolebole in collecting paired blood samples were linked particularly to a chaotic context. In a more relaxed situation, collecting paired samples must be a priority because quality sputum specimens will remain difficult to obtain in the field. For the same reason, blood cultures could be attempted. This would require specific transport media, but this is not an issue for a national or international intervention. To date, according to the WHO case definition, neither PCR nor DFA is established as a confirmatory test. Both techniques have limitations and can be affected by the specimen conditions or a prior administration of antimicrobial drugs. However, positive results can reinforce the suspicion and contribute to the understanding of the outbreak. Both should be considered an important part of the biological investigation when plague emergence is suspected.

Conclusions

Pneumonic plague emerged in new locations in the Oriental Province of DRC with outbreaks in 2005 and 2006. Due to the remoteness and political instability in this region, access to these locations was constrained, which added to the challenges in managing these outbreaks and confirming their cause. Compared with the first outbreak, the second outbreak showed that the detection of the F1-antigen by RDT was able to strengthen the frontline response strategy. In this experience, the RDT test proved to be an important step for preliminary diagnosis, which led to an early alert of the outbreak and facilitated the implementation of the first control measures.

However, in previously unknown plague foci, the isolation of *Y. pestis* remains the absolute proof of the outbreak cause. The importance of a well-prepared and well-equipped strategy was demonstrated in 2006. It can be summarized as an empowered frontline and a robust reference laboratory with efficient transportation and minimized handling between the two. In 2005 and 2006, an international reference laboratory was essential. Today, in similar situations of emergence of new plague foci, strengthening the national plague reference laboratory would likely limit the need for international transportation of potentially dangerous specimens to only the first confirmations. This effort should be extended to all the plague-endemic countries in Africa.

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Evolution of New Genotype of West Nile Virus in North America

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Previous studies of North American isolates of West Nile virus (WNV) during 1999–2005 suggested that the virus had reached genetic homeostasis in North America. However, genomic sequencing of WNV isolates from Harris County, Texas, during 2002–2009 suggests that this is not the case. Three new genetic groups have been identified in Texas since 2005. Spread of the southwestern US genotype (SW/WN03) from the Arizona/Colorado/northern Mexico region to California, Illinois, New Mexico, New York, North Dakota, and the Texas Gulf Coast demonstrates continued evolution of WNV. Thus, WNV continues to evolve in North America, as demonstrated by selection of this new genotype. Continued surveillance of the virus is essential as it continues to evolve in the New World.

West Nile virus (WNV) is a mosquito-borne flavivirus belonging to the Japanese encephalitis serogroup and maintained in an enzootic cycle between mosquitoes (primarily *Culex* spp.) and birds. Mammals such as horses and humans act as dead-end hosts. Most human infections are asymptomatic; West Nile fever develops in ≈20% of infected patients and neuroinvasive disease develops in <1% (*1*).

WNV was first isolated in Uganda in 1937 and was generally associated with sporadic outbreaks of mild, febrile illness until the 1990s, when several epidemics of neuroinvasive disease were reported in northern Africa, eastern Europe, and Russia (*2*–*4*). In 1999, WNV was first isolated in North America from human and bird samples during an outbreak of encephalitic disease in New York. After this outbreak, WNV rapidly spread across the United States north to Canada and south to the Caribbean region, Mexico, and Central and South America.

By 2002, the original WNV genotype isolated in New York, known as NY99, was displaced by a new genotype, designated the North American (NA) or WN02 genotype (hereafter termed NA/WN02 genotype) (*5*,*6*). This genotype is characterized by 13 conserved nt changes, 1 of which results in an amino acid substitution, V159A, in the envelope (E) protein. The NA/WN02 genotype is believed to have become dominant in North America because of its ability to more efficiently disseminate in mosquitoes than the original NY99 virus genotype (*6*–*8*).

Beasley et al. (*9*) first identified the NA/WN02 genotype in Texas in 2002, and further studies showed that this genotype had spread throughout the Upper Texas Gulf Coast and to other regions in the United States (*5*). Additional studies examined phenotypic changes in WNV isolates from the Upper Texas Gulf Coast region during 2003 and identified co-circulation of small-plaque, temperature-sensitive, mouse-attenuated and large-plaque, non–temperature-sensitive, mouse-virulent strains (*10*–*12*). Subsequent studies of the E gene of viruses isolated through 2006 suggested that since the emergence of the NA/WN02 genotype, WNV in North America is either genetically homeostatic (*13*) or its growth rate is decreasing (*14*).

We examined genetic variation in selected WNV strains since 2005 from the Upper Texas Gulf Coast region, in particular, Harris County, Texas, USA (Houston metropolitan area). We report the isolation of genetic variants that demonstrate the continuing evolution of WNV in North America. We also show that the southwestern US genotype first identified in Arizona, Colorado, and northern Mexico in 2003 (termed SW/WN03 genotype) has now spread to the Upper Texas Gulf Coast region.

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Materials and Methods

Virus Isolates

Virus isolates were obtained from the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch (UTMB) in Galveston, Texas. All new isolates used in this study were originally made from mosquito pools or the brains of naturally infected birds cultured in Vero cells at UTMB. Each isolate was given a second passage in Vero cells to generate a working stock and stored at –80°C.

Reverse Transcription–PCR

Viral RNA was extracted from 140 μL of infected Vero cell supernatant by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) per the manufacturer's directions. Full-genome sequencing was performed by consensus overlapping sequencing of PCR products with primers based on the published sequence of WNV NY-99 flamingo 382–99 (GenBank accession no. AF196835). Reverse transcription–PCR was performed by using the Titan One Tube RT-PCR Kit (Roche Applied Science, Indianapolis, IN, USA) (primers and PCR conditions are available by request). PCR products were subjected to electrophoresis on 1% agarose gels and purified by using the QIAquick Gel Extraction Kit (QIAGEN).

Sequencing and Analysis

Purified PCR products were sequenced in both directions by using the Protein Chemistry or Molecular Genomics Core Laboratories at UTMB. Sequences were edited and assembled by using ContigExpress in the VectorNTI program suite (Invitrogen, Carlsbad, CA, USA). Full-length coding sequences were aligned with all published full-length North American WNV isolate sequences available in GenBank (as of November 2010) and isolate WNV IS-98 STD by using MUSCLE in Seaview version 4 (*15*). The final open reading frame (ORF) alignment contained 244 sequences of 10,299 nt (3,433 aa residues). A second alignment was made by using MUSCLE; this aligment contained 33 sequences from the Upper Texas Gulf Coast region. This alignment contained 11,030 nt and contained the entire ORF and portions of the 3′ and 5′ untranslated region (UTR).

Phylogenetic trees were inferred using the neighbor-joining (NJ) method in the Phylip package (*16*) and the maximum-likelihood (ML) method by using PhyML (*17*). MODELTEST, in conjunction with PAUP, was used to identify generalized time reversible + I + Γ_4 as the best-fit nucleotide substitution model to be used in phylogenetic analyses (*18*,*19*). To assess robustness of the phylogenetic methods used, we used the NJ method and 1,000 bootstrap replicates. The ML method used 100 bootstrap replicates for the entire North American alignment and 1,000 bootstrap replicates for the Upper Texas Gulf Coast region alignment. IS-98 STD was used as outgroup for the entire North American WNV alignment, and NY99 was used as outgroup for the Upper Texas Gulf Coast region alignment.

Recombination Detection and Selection Analysis

Screening for recombination was performed on the first 9,999 nt of the North American WNV ORF alignment by using single-break point analysis on the Datamonkey server (*20*–*22*). This screening verified absence of recombination in sequences before running the selection analyses. The first 9,999 nt were selected because of constraints on sequence length by the programs used.

Using the ratio of nonsynonymous (d_N) to synonymous (d_S) nucleotide substitutions, we examined the genome for sites of positive selection. Positive selection was defined as $d_N > d_S$ and a p value <1.0. Using the Datamonkey web server (*21*,*22*), we used 3 methods to detect site specific nonneutral selection: single-likelihood counting (SLAC), fixed effects likelihood (FEL), and internal FEL (IFEL) (*23*,*24*). BioEdit was used to create datasets for the first 9,999 nt of the ORF and for each gene (capsid [C], premembrane [prM], E, nonstructural protein 1 [NS1], NS2A, NS2B, NS3, NS4A, NS4B, and NS5) for these analyses (*25*).

Results

Viral Isolates

Viruses used in this study were isolated during 2005–2009 from mosquitoes or dead birds collected in Harris County. There were 111 isolates: 14 from 2005, 11 from 2006, 36 from 2007, one from 2008, and 49 from 2009. The genomic sequences of 17 geotemporally representative isolates were determined and compared with other WNV strains from the Upper Texas Gulf Coast region (Harris, Jefferson, and Montgomery Counties) isolated during 2002–2005 and sequenced in our laboratory (Table 1) (*5*,*10*,*12*).

Harris County Isolates, 2005–2009

Nucleotide Changes

The genome of WNV is a single-stranded, positive-sense RNA molecule; the NY99 strain contains 11,029 nt. The genome encodes a 5′ UTR (genomic nt 1–96) and a 3′ UTR (genomic nt 10,396–11,029). The UTRs flank a single ORF that encodes 10 proteins; 3 structural proteins (C, prM/M, and E) and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).

When compared with NY99, the prototype WNV strain for North America, the 17 WNV isolates we analyzed in this study had 38–60 nt (0.35%–0.54%) differences;

Table 1. West Nile viruses from the Upper Texas Gulf Coast, USA, used to study genotype evolution, 2002–2009

Strain	Source	County	Collection year	GenBank accession no.
TX2002–1	Human	Unknown	2002	DQ164198
TX2002–2	Human	Unknown	2002	DQ164205
TVP8533	Human	Jefferson	2002	AY218294
Bird 114	Blue jay	Harris	2002	GU827998
Bird1153	Mourning dove	Harris	2003	AY712945
Bird1171	Great-tailed grackle	Harris	2003	AY712946
v4095	*Culex quinquefasciatus* mosquito	Harris	2003	GU828002
v4380	*Cx. quinquefasciatus* mosquito	Harris	2003	GU828001
Bird1881	Mourning dove	Jefferson	2003	GU828003
Bird1519	Blue jay	Montgomery	2003	GU828004
Bird1576	Blue jay	Montgomery	2003	GU827999
Bird1175	Blue jay	Harris	2003	GU828000
Bird1461	Blue jay	Harris	2003	AY712947
v4369	*Cx. quinquefasciatus* mosquito	Harris	2003	AY712948
Bird3588	Blue jay	Harris	2004	DQ164206
TX5058	Blue jay	Harris	2005	JF415929
M12214	*Cx. quinquefasciatus* mosquito	Harris	2005	JF415915
TX5810	Common grackle	Harris	2006	JF415916
M6019	*Cx. quinquefasciatus* mosquito	Harris	2006	JF415930
TX6276	Northern mockingbird	Harris	2006	JF415916
TX6647	Blue jay	Harris	2007	JF415917
TX6747	Blue jay	Harris	2007	JF415918
M19433	*Aedes albopictus* mosquito	Harris	2007	JF415919
TX7191	Blue jay	Harris	2007	JF415920
TX7558	Blue jay	Harris	2008	JF415921
M37012	*Cx. quinquefasciatus* mosquito	Harris	2009	JF415922
M37906	*Cx. quinquefasciatus* mosquito	Harris	2009	JF415923
TX7827	Blue jay	Harris	2009	JF415924
M38488	*Ae. albopictus* mosquito	Harris	2009	JF415925
M20140	*Cx. quinquefasciatus* mosquito	Harris	2009	JF415926
M20141	*Ae. albopictus* mosquito	Harris	2009	JF415927
M20122	*Cx. quinquefasciatus* mosquito	Harris	2009	JF415928

most changes were synonymous. Nine of the 13 conserved nt changes characteristic of the NA/WN02 genotype were found in all newly sequenced isolates (Table 2). One 2006 isolate (TX6276), two 2007 isolates (TX6747 and TX7191), and six of seven 2009 isolates (M37012, M37906, M39488, M20140, M20141, and M20122) encoded a C at nt 660 and 6238, which was identical to that in the NY99 strain. Two isolates (M12214 from 2005 and M19433 from 2007) encoded a C at nt 6426, and 2 isolates (TX5810 and M6019, both from 2006) encoded a U at nt 9352, again identical to the NY99 strain. One 2005 isolate (TX5058) contained a 6-nt (nt 10471–10476) deletion in the 3′ UTR, and one 2007 isolate (TX7191) and two 2009 isolates (M37906 and TX7827) contained a 1-nt deletion at nt position 49/50 in the 5′ UTR.

Amino Acid Substitutions

Deduced amino acid sequences were compared and substitutions were identified for 41 residues (2 in C, 4 in prM/M, 6 in E, 0 in NS1, 6 in NS2A, 2 in NS2B, 8 in NS3, 2 in NS4A, 4 in NS4B, and 7 in NS5); each isolate contained 3–7 substitutions (online Appendix Table 1, www.cdc.gov/EID/content/17/5/785-appT1.htm.). Eight (C-T109I, E- T70I, E-V159A, E-I460M/L, NS2A-R98G, NS2A-A137V, NS4A-A85T, and NS4B-I240M) of the 41 substitutions were found in >1 isolate. All isolates contained the E-V159A substitution present in the NA/WN02 genotype.

Upper Texas Gulf Coast Region Isolates, 2002–2009

WNV was first detected in the Upper Texas Gulf Coast region in 2002 (*9*). During 2002–2004, isolates from the Upper Texas Gulf Coast region were divided genetically into 3 groups (groups 1–3) (*12*) and showed 0.30%–0.40% divergence compared with NY99. Isolates from 2005–2009 (groups 4–6; see below for their definitions) have significantly greater divergence (0.40%–0.70%; $p<9.4 \times 10^{-9}$) from NY99 (Table 3). When compared with isolates from 2002–2004 (groups 1–3), we found that recent Upper Texas Gulf Coast isolates from 2005–2009 (groups 4–6) have nucleotide divergence rates ranging from 0.50% to 0.80%. Because of the high number of synonymous nucleotide mutations, the deduced amino acid sequences of all isolates exhibited a higher level of conservation;

Table 2. Nucleotide sequence changes in West Nile virus NA/WN02 genotype, Upper Texas Gulf Coast, USA*

		prM	E		NS2A		NS3				NS4B	NS5		3′ UTR
Strain	Year	660	1442	2466	3774	4146	4803	6138	6238	6426	6996	7938	9352	10851
NY99	1999	C	U	C	U	A	C	C	C	C	C	U	C	A
Bird 114	2002	U	C†	U	U	G	U	U	U	U	U	C	U	G
M12214	2005	U	C	U	U	G	U	U	U	·	U	C	U	G
TX5058		U	C	U	U	G	U	U	U	U	U	C	U	G
TX5810	2006	U	C	U	U	G	U	U	U	U	U	C	·	G
M6019		U	C	U	U	G	U	U	U	U	U	C	·	G
TX 6276		·	C	U	U	G	U	U	·	U	U	C	U	G
TX 6647	2007	U	C	U	U	G	U	U	U	U	U	C	U	G
TX6747		·	C	U	U	G	U	U	·	U	U	C	U	G
M19433		U	C	U	U	G	U	U	U	·	U	C	U	G
TX7191		·	C	U	U	G	U	U	·	U	U	C	U	G
TX 7558	2008	U	C	U	U	G	U	U	U	U	U	C	U	G
M 37012	2009	·	C	U	U	G	U	U	·	U	U	C	U	G
M 37906		·	C	U	U	G	U	U	·	U	U	C	U	G
TX 7827		U	C	U	U	G	U	U	U	U	U	C	U	G
M 39488		·	C	U	U	G	U	U	·	U	U	C	U	G
M 20140		·	C	U	U	G	U	U	·	U	U	C	U	G
M 20141		·	C	U	U	G	U	U	·	U	U	C	U	G
M20122		·	C	U	U	G	U	U	·	U	U	C	U	G

*PrM, premembrane; E, envelope; NS, nonstructural; UTR, untranslated region. Values indicate nucleotide position within each gene. Dots indicate no change from NY99 isolate.
†Encodes for amino acid substitution E-V159A.

divergence rates ranged from 0.10% to 0.30% compared with NY99.

With the exception of conserved nucleotide mutations in the NA/WN02 genotype, there were a few nucleotide changes or deduced amino acid substitutions that were shared by ≥1 isolate from 2002–2004 and 1 of the newly sequenced isolates from 2005–2009. Nucleotide changes at 11 positions were shared between ≥1 isolate from 2002–2004 and the newly sequenced isolates from 2005–2009, with only 1 aa substitution, NS4B-I240M, found in ≥1 isolate from both groups.

Phylogenetic Analysis

Phylogenetic trees were generated by NJ and ML analyses by using only the polyprotein sequence of 34 isolates: the 17 newly sequenced isolates, 16 published sequences of isolates from the Upper Texas Gulf Coast region, and NY99 (Figure 1). Both methods produced trees with similar topology. In addition to groups 1, 2, and 3 identified in Upper Texas Gulf Coast region isolates obtained in 2002–2003 (*12*), we identified 3 other phylogenetic groups in this study.

Group 4 is composed of 6 of 7 isolates from 2009 and 1 isolate from 2006 (TX6276). Group 5 is composed of 4 isolates: 1 from 2005 (M12214), 2 from 2007 (M19433 and TX6647), and 1 from 2008 (TX7558). Group 6 is composed of 2 isolates from 2006 (M6019 and TX5810) and 2 sequenced isolates from 2002. Groups 4 and 5 are supported by high bootstrap values; group 6 has a lower bootstrap value. Within group 4, all 2009 isolates contained an E-I460E substitution. All group 5 isolates contain the amino acid substitution NS4B-A85T, and all isolates in groups 4 and 6 contain the NS4B-I240M substitution. Four isolates did not fall into these 6 groups: TX5058 (2005), TX6747 and TX7191 (2007), and TX7827 (2009).

Table 3. Percentage nucleotide and amino acid sequence divergence of West Nile virus isolates, Upper Texas Gulf Coast, USA, 2002–2009*

Group†	NY99	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
NY99		0.1–0.2	0.1–0.2	0.1	0.1–0.2	0.2	0.2–0.3
Group 1	0.3–0.4		0.1–0.3	0.1–0.3	0.1–0.3	0.2–0.3	0.2–0.4
Group 2	0.3	0.3–0.4		0.1–0.2	0.2–0.3	0.2–0.3	0.2–0.3
Group 3	0.3	0.1–0.4	0.2–0.3		0.1–0.3	0.2–0.3	0.2–0.3
Group 4	0.5–0.6	0.5–0.7	0.5–0.6	0.5–0.6		0.2–0.3	0.2–0.3
Group 5	0.5–0.7	0.5–0.8	0.4–0.7	0.4–0.7	0.7–1.0		0.3
Group 6	0.4–0.5	0.4–0.6	0.4–0.5	0.4–0.5	0.7–0.8	0.6–0.8	

*Amino acid sequence divergence is shown above the diagonal, and nucleotide sequence divergence is shown in below the diagonal.
†Group 1: bird 114 (2002), bird 1171 (2003), bird 1153 (2003); group 2: bird 1519 (2003), v4369 (2003), v4095 (2003), bird 1881 (2003), v4380 (2003); group 3: bird 1576 (2003), bird 1175 (2003), TX2003; group 4: TX6376 (2006), M20141 (2009), M20140 (2009), M37906 (2009), M39488 (2009), M20122 (2009), M37102 (2009); group 5: M12214 (2005), M19433 (2007), TX6647 (2007), TX7558 (2008); group 6: M6019 (2006), TX5810 (2006).

The TX7828 2009 isolate, which did not fall into group 4 with the other 2009 isolates, is the only 2009 isolate sequenced in this study that was isolated from a bird (blue jay). However, we had only 1 isolate from 2008, and WNV activity was low in Harris County in 2008 (R. Bueno and R. Tesh, unpub. data). This finding may have been caused by Hurricane Ike, which hit the Upper Texas Gulf Coast in September 2008.

A second phylogenetic analysis was undertaken that used isolates from this study and all published full-length WNV sequences from North America available on GenBank (Figure 2). NJ and ML methods produced trees with similar topology. Within the larger tree, there were analogous groupings of previously and newly sequenced Harris County isolates, as shown in Figure 1. Three of the Harris County groups form distinct clusters of isolates within the NA/WN02 genotype and may represent formation of new genotypes or clusters. Group 1 Harris County isolates (2002–2003) cluster with a grouping of isolates from California from 2003–2008, and group 4 isolates (2006–2009) cluster with several isolates from New York (2008) and 1 isolate from Illinois (2006). Group 5 isolates from Harris County cluster with isolates from the southwestern United States and northern Mexico (called the SW/WN03 genotype because the first isolates were identified in Arizona and Colorado in 2003).

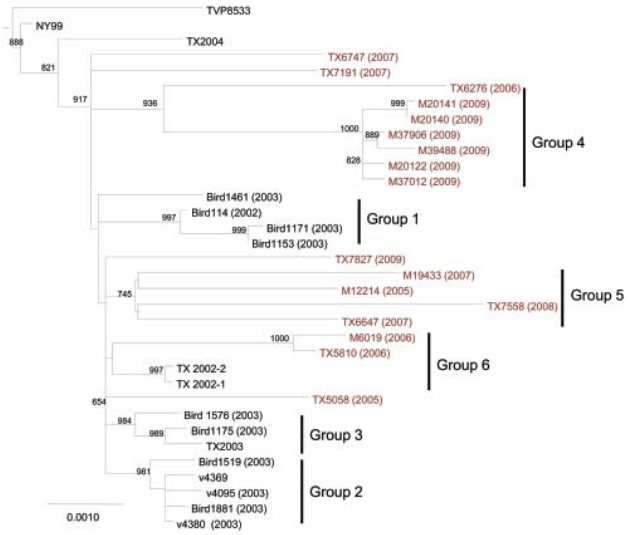

Figure 1. Maximum-likelihood phylogenetic tree of Upper Texas Gulf Coast, USA, West Nile virus isolates, 2002–2009. The tree was inferred from open reading frame sequences of 33 Upper Texas Gulf Coast isolates and NY99 by using PhyML (*17*) and rooted with IS-98 STD. The outgroup has been removed. Bootstrap values are for 1,000 replicates and only values >500 are shown. Groups 1–3 were previously identified by May et al. (*12*). Red, isolates sequenced in this study. Scale bar indicates nucleotide substitutions per site.

SW/WN03 Genotype

This genotype is composed of 5 groups on the basis of nucleotide and amino acid sequences and phylogenetic analysis. SW Group 1 is composed of 2 isolates: WNV-1/US/BID-V4093/2007 from New York and CO2003–2 from Colorado. SW Group 2 is composed of 4 isolates, 3 from Texas that were sequenced in this study: M12214 (2005), TX6647 (2007), TX7558 (2008), and BSL2–05 from South Dakota in 2005. SW Group 3 is composed of 6 isolates: 2 from Illinois (WNV-1/US/BID-V4369/2004 and WNV-1/US/BID-V4378/2005), 2 from New York (WNV-1/US/BID-V4806/2005 and WNV-1/US/BID-V4624/2008), and 2 from New Mexico (04–237NM and 04–238NM). SW Group 4 is composed of 8 isolates from Mexico in 2003 (TVP9115, TVP9118–TVP9222). SW Group 5 is the largest and is composed of 22 isolates: 10 from Arizona (2003–2006), 2 from New Mexico (2005), 2 from Colorado (2004), 3 from California (2005, 2007–2008), and 5 from Texas (M19433, which was sequenced in this study, and 4 isolates from west Texas).

Further examination of sequences within the SW/WN03 genotype showed that they share some or all of a signature of 13 nt changes (different from those of the NA/WN02 genotype), including 2 aa substitutions, NS4B-A85T and NS5-K314R (online Appendix Table 2, www.cdc.gov/EID/content/17/5/785-appT2.htm). Isolates in SW group 1 contain 4 of the 13 changes (nt positions 6238, 6721, 7269, and 9264). SW group 2 isolates contain 5 changes (nt positions 6238, 6721, 8550, 9264, and 9660). SW group 3 isolates have 7 changes (nt positions 1320, 6238, 6721, 8550, 8621, 9264, and 9660). SW group 4 isolates have 6 changes (nt positions 1320, 6238, 6721, 8550, 8621, and 9660). SW group 5 isolates have all 13 nt changes (nt positions 1320, 1974, 3399, 6238, 6721, 6765, 6936, 7269, 8550, 8621, 9264, 9660, and 10062). Isolates in SW groups 1 and 2 contain only 1 (NS4B-A85T) of the 2 aa substitutions, and isolates in SW groups 3, 4, and 5 contain both amino acid substitutions.

Selection Pressures

Recombination analysis using single-break point analysis was performed on the first 3,333 codons of the ORF of the North American WNV alignment to rule out recombination before performing selection pressure analysis. As expected, no evidence of recombination was detected.

Selection pressures on the WNV genome were examined by using 3 methods: SLAC, FEL, and IFEL (Table 4). These methods estimated the ratio of nonsynonymous (d_N) to synonymous (d_S) amino acid substitutions in 10 datasets representing the first 9,999 nt (3,333 aa residues)

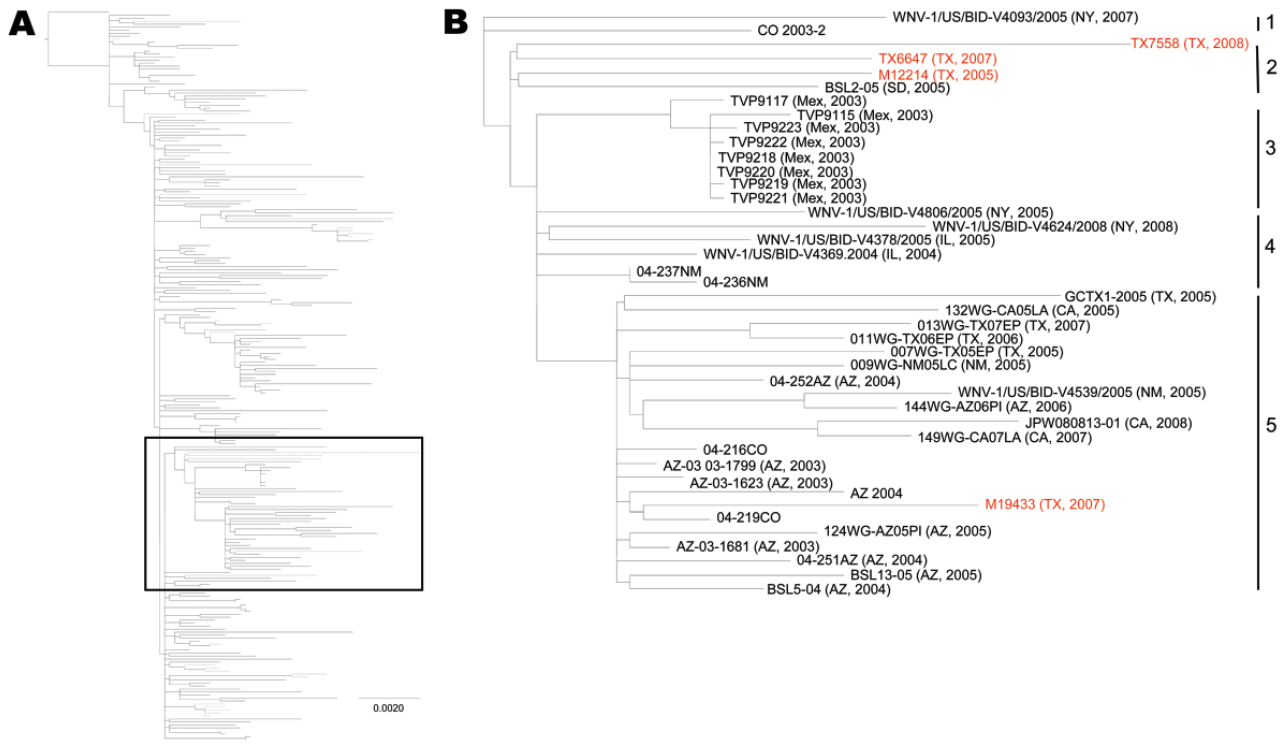

Figure 2. Maximum-likelihood phylogenetic tree showing all published, full open reading frame North American West Nile virus isolates, 2002–2009 (A), and enlargement showing SW/WN03 genotype (B). Red, isolates sequenced in this study. Scale bar in panel A indicates nucleotide substitutions per site. Numbers on the right in panel B indicate groups.

of the ORF and each protein (C, prM/E, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) for the complete North American WNV alignment of 244 genomes. On examination of the ORF, 3 residues were identified for positive selection in ≥2 of the 3 methods. E-V431I (codon position 721 in ORF) was identified by FEL (p = 0.057) and IFEL (p = 0.072), NS2A-A224V/T (codon position 1367 in ORF) was identified by SLAC (p = 0.087) and FEL (p = 0.096), and NS4A-A85T (codon position 2209 in ORF) was identified by SLAC (p = 0.087), FEL (p = 0.011), and IFEL (p = 0.067). When selection analysis was performed on each gene, only E-V431I was identified for positive selection (FEL, p = 0.059 and IFEL, p = 0.065). An additional residue, NS5-K314R, was also identified for positive selection by FEL (p = 0.042) and IFEL (p = 0.042).

NS4A-A85T and NS5-K314R are the 2 aa residues that identify the SW/WN03 genotype described. Residue E-V431I is found in a California cluster (California isolates 2003–2008). Substitutions at NS2A-224 (codon position 1367 in ORF) are found in 5 NY99 genotype isolates (A224T) and 4 SW/NA03 genotype isolates (A224V).

Discussion

To date, most genetic and phylogenetic studies of WNV have focused on partial genome sequencing, primarily of the E protein gene. Although studies of the E protein gene are helpful in understanding the evolution of WNV in North America, they provide few phylogenetically informative sites; analysis of genomic sequences is more informative (*5*,*26*–*32*). Similarly, although many studies have examined the evolutionary dynamics of WNV soon after its introduction into North America, only 1 published study has examined isolates since 2006 (*33*). To our knowledge, none have been published that examined isolates from 2007 or more recently. For these reasons, we examined evolution of WNV by using genomic sequences from 1999–2009. We focused on the Upper Texas Gulf Coast region because of availability of multiple isolates from the same localities each year since the first detection of the virus in Texas in 2002. These isolates were obtained as part of an ongoing surveillance program of WNV activity in Harris County.

The isolates sequenced in this study demonstrate that the NA/WN02 genotype has been maintained during 2002–2009 in Harris County. All 17 isolates sequenced contained 9 of 13 nt changes associated with the NA/WN02 genotype reported by Davis et al. (*5*), including the amino acid substitution E-V159A. However, since 2005, reversion to the NY99 genotype was seen at 4 nt positions. Nine isolates contained a C at nt positions 660 and 6238, three isolates

Table 4. Positive and negative selection results for West Nile virus isolates, Upper Texas Gulf Coast, USA, 2002–2009*

Protein	Amino acid residues relative to ORF	Length of protein, aa	Overall d_N/d_S	Single-likelihood ancestor counting†		Fixed effects likelihood†		Internal fixed effects likelihood†	
				Positive selection	Negative selection	Positive selection	Negative selection	Positive selection	Negative selection
ORF	1–3,333‡	3,333	0.110	2	246	8	619	16	25
C	1–123	123	0.270	0	2	0	9	0	2
prM	124–290	166	0.134	0	7	0	25	1	3
E	291–791	500	0.119	0	15	1	72	1	3
NS1	792–1143	351	0.134	0	17	1	48	4	4
NS2A	1144–1374	230	0.130	0	15	1	48	1	3
NS2B	1375–1505	130	0.118	0	6	0	19	0	1
NS3	1506–2124	618	0.083	0	42	0	101	0	6
NS4A	2125–2273	148	0.135	0	7	1	22	0	3
NS4B	2274–2522	248	0.112	0	13	0	49	0	8
NS5	2529–3433	904	0.098	0	61	2	148	4	10

*ORF, open reading frame; d_N, nonsynonymous nucleotide substitutions; d_S, synonymous nucleotide substitutions; C, capsid; prM, premembrane; E, envelope; NS, nonstructural.
†No. sites where p<0.1.
‡Only the first 3,333 aa residues of the ORF were used for these analyses because of program constraints on alignment size.

had a U at nt position 6426, and two isolates had a C at nt position 9352.

Although isolates sequenced in our study display a high degree of similarity, they have major differences. It appears that ≥3 genetic groups of isolates were co-circulating in Harris County over the study period. Thus, there is continued genetic diversity of WNV over time, at least in the Upper Texas Gulf Coast region, rather than the genetic homeostasis in North America, which was proposed on the basis of using E gene sequences of viruses isolated through 2005 (*13*). One group, group 4, contains isolates from 2006 and 2009. A second group, group 5, contains isolates from 2005, 2007, and 2008. A third group, group 6, contains isolates from 2006 plus 2 sequenced isolates from 2002. All other 2002–2005 isolates sequenced previously fall into other groups (groups 1–3) (*12*). Four isolates, TX5058 from 2005, TX6747 and TX7191 from 2007, and TX7827 from 2009, did not fall into any of the 6 groups and may represent single isolates that did not have any advantage and thus became extinct.

When compared with all North American WNV isolates, we found 3 distinctive clusters of isolates within the NA/WN02 genotype. Each cluster contained several isolates from the Upper Texas Gulf Coast region, in addition to other isolates. The first cluster contains group 4 isolates, in addition to four 2008 isolates from New York and one 2006 isolate from Illinois. The second cluster is composed primarily of isolates from California, in addition to group 1 Upper Texas Gulf Coast isolates and 3 additional isolates from Colorado, Connecticut, and Illinois. Group 5 isolates from Harris County cluster with isolates from the southwestern United States (Arizona and Colorado) and from northern Mexico, which were first identified in 2003. This SW/WN03 genotype shares some or all of 13 nt changes, which encode for 2 aa substitutions.

Our data indicate that this genotype is spreading into new areas. It has been identified in California, Illinois, New Mexico, New York, North Dakota, and Texas since 2003. Two of these clusters, the California cluster and the cluster we have called the SW/WN03 genotype, are further supported by using selection analysis. This analysis has shown that there is potential for positive selection at E-V431I in the California cluster and at both of the amino acid substitutions (NS4A-A85T, NS5-K314R) in the SW/WN03 genotype. This finding further provides evidence of the potential role of this emerging genotype.

Potential roles of single amino acid substitutions within the WNV genome should also be noted. The single amino acid change, E-V159A, which occurred in the NA/WN02 genotype, was shown to decrease the extrinsic incubation period of the virus in mosquitoes, which enabled that genotype to displace the NY99 genotype (*6*). Brault et al. (*34*) reported that the NS3-T249P substitution increased virulence in American crows. The NS3-T249P substitution has undergone positive selection but the E-V159A change has not, yet both cause phenotypic changes. We speculate that positive selection of NS4A-A85T and NS5-K314R induces a phenotypic change in WNV.

Previous studies in our laboratory that focused on the E protein gene concluded that WNV is experiencing a genetic stasis or decrease in its growth rate after establishment of the NA/WN02 genotype (*13*). However, none of these studies have phylogenetically examined the entire genome of WNV. This study of genomic sequences demonstrates evolution of WNV, at least in the Upper Texas Gulf Coast region, and potential emergence of a new genotype in the southwestern United States (SW/WN03 genotype). Further experiments are needed to investigate potential phenotypic changes that occur in conjunction with the noted genotype changes and to determine if the

SW/WN03 genotype will replace the current dominant NA/WN02 genotype.

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Ms McMullen is a predoctoral student at the University of Texas Medical Branch. Her research interests include the pathogenesis and molecular epidemiology of flaviviruses.

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Transstadial Transmission of *Francisella tularensis holarctica* in Mosquitoes, Sweden

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In Sweden, human cases of tularemia caused by *Francisella tularensis holarctica* are assumed to be transmitted by mosquitoes, but how mosquito vectors acquire and transmit the bacterium is not clear. To determine how transmission of this bacterium occurs, mosquito larvae were collected in an area where tularemia is endemic, brought to the laboratory, and reared to adults in their original pond water. Screening of adult mosquitoes by real-time PCR demonstrated *F. tularensis lpnA* sequences in 14 of the 48 mosquito pools tested; *lpnA* sequences were demonstrated in 6 of 9 identified mosquito species. Further analysis confirmed the presence of *F. tularensis holarctica*–specific 30-bp deletion region sequences (FtM19inDel) in water from breeding containers and in 3 mosquito species (*Aedes sticticus*, *Ae. vexans*, and *Ae. punctor*) known to take blood from humans. Our results suggest that the mosquitoes that transmit *F. tularensis holarctica* during tularemia outbreaks acquire the bacterium already as larvae.

Outbreaks of tularemia are caused by the bacterium *Francisella tularensis holarctica* throughout the Northern Hemisphere and by *F. tularensis tularensis* in North America only. Routes of infection include transmission from blood-sucking arthropods and through contact with infected dead or live animals, as well as from aerosols, dust, and water (*1*). Two primary disease manifestations, ulceroglandular and glandular tularemia, are associated with vector-borne transmission of the bacterium (*1*). Traditionally, mosquitoes are considered the primary vectors of *F. tularensis holarctica* to humans in Russia and Scandinavia (*2*–*4*,*5*). Moreover, mosquito-borne transmission of tularemia may be becoming more common in central Europe; evidence shows that this infection has reemerged during the past decade (*6*,*7*).

The ulceroglandular form of tularemia is by far the most common in Sweden; most human cases occur in late summer and early fall and are assumed to be transmitted by mosquitoes (*4*,*8*). A total of 5,754 human cases of tularemia were reported during 1931–1993, and the incidence of infection varies greatly among these years, ranging from a few cases in some years to >2,700 cases during 1967 (*8*). In the Örebro area of central Sweden, widespread mosquito-associated tularemia outbreaks first occurred during 2000 and 2003 (*4*,*5*), after which human cases have continued to occur in this new area where tularemia is endemic (www.smi.se/statistik/harpest). However, how vector mosquitoes acquire the bacterium is still not clear.

The demonstrated ability of *F. tularensis holarctica* strains to survive in association with protozoa indicates that ubiquitous aquatic protozoa might be an important environmental reservoir for the bacterium (*9*–*11*). Moreover, mosquito larvae, mainly the species *A. sticticus* and other floodwater mosquitoes, exert a predatory effect on aquatic protozoan populations (*12*). These factors indicate that mosquito larvae may be exposed to *F. tularensis* in their natural aquatic environment. We investigated the natural occurrence of *F. tularensis* in mosquitoes hatched from larvae collected in an area where tularemia was endemic. Because of unknown mechanisms, the bacterium

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F. tularensis is extremely difficult to isolate directly from environmental samples. Thus, our study focuses entirely on molecular techniques.

Materials and Methods

Sample Collection

Mosquito larvae were sampled on August 28, 2008, in Örebro, an area where tularemia is endemic. Nine human tularemia cases (3.24/100,000 persons) were reported from Örebro County in June–September 2008 (www.smi.se/statistik/harpest). Two sampling locations were selected, Ormesta (WGS84; 59°16′12″N, 15°16′48″E) and Vattenparken (WGS84; 59°16′55″N, 15°15′00″E), on the basis of a geographic distribution study of human tularemia cases in the area (*13*). Both locations are situated at Lake Hjälmaren near the city of Örebro and are characterized by lush vegetation of reed belts and deciduous trees and bushes. Using a standard dipper, we collected mosquito larvae from shallow temporary water bodies in the transition zone between reed and willow bush habitats (Ormesta 1), in the deciduous forest (Ormesta 2), and in a ditch covered by bush and grass (Vattenparken).

Mosquito larvae from each water body were reared to adults in their original pond water (Ormesta 1, containers A and B; Ormesta 2, containers C, D, E, F, and G; and Vattenparken, container H). At the start of this study, time-zero samples of the original water from each container were collected and stored at –20°C. Emerging adult mosquitoes were collected by a mechanical aspirator, killed by freezing, and stored at –80ºC until species was identified. During identification, adult mosquitoes were kept cold on a chill table, illuminated by a cold light lamp, and identified to species based on morphologic features. Identified mosquitoes were sorted by area, species, and sex in pools of 1–50 specimens and returned to the –80ºC freezer.

DNA Extraction from Mosquitoes and Water Samples

For DNA extraction, 10 μL of 2.8 M NH_4OH solution and 450 mg each of 1.0-mm and 0.1-mm silica beads were added to each pooled mosquito sample. Samples were homogenized for 60 s (BeadBeater FastPrep; BioSpec Products, Inc., Bartlesville, OK, USA). The homogenized samples were incubated at room temperature for 15 min, and 60 μL of sterile water was added. DNA extraction was performed by using SoilMaster DNA Extraction Kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. The resulting DNA pellet was resuspended in 60 μL Tris EDTA buffer.

DNA extraction from water samples was performed as previously described (*14*). Two milliliters of each water sample was centrifuged at 16,000 × *g* for 1 h, 1.9 mL of the resulting supernatant was discarded, and DNA was extracted from the remaining volume by using a SoilMaster DNA Extraction Kit (Epicentre Biotechnologies).

PCR Analysis of Mosquitoes and Water

Mosquitoes and water samples were screened for *F. tularensis* by using a modified real-time PCR SYBR-based assay (Quanta BioSciences, Gaithersburg, MD, USA) for detection of the *F. tularensis*-specific *lpnA* gene. The PCR assay was modified from the methods of Thelaus et al. (*11*). Each reaction consisted of 1 μL template, 1× Quanta PerfeCTa SYBR Green FastMix (Quanta BioSciences), 400 nmol/L for each of the *lpnA*2F/R-primers (5′-CGCAGGTTTAGCGAGCTGTT-3′ and 5′-GAGCAGCAGCAGTATCTTTAGC-3′), and Milli-Q (Millipore, Billerica, MA, USA) up to 20 μL. An initial denaturation at 95°C for 5 min was followed by 40 cycles at 95°C for 10 s, 60°C for 30 s, and a melt curve 60°C–95°C on Mastercycler (Eppendorf, Hamburg, Germany).

Mosquitoes and water samples then underwent a *F. tularensis holarctica*–specific PCR, based on the 30-bp–deletion region FtM19 and using the FtM19InDelF/R primer pair, and modified from PCR (*14*). Each reaction consisted of 1–3 μL templates, 1x SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) 400 nmol/L for each of the primers FtM19Indel F/R (5′-GAATTACATAAAGTTCATGGTCCAGTAC-3′ and 5′-GTTTCAGAATTCATTTTTGTCCGTAA-3′) and Milli-Q (Millipore) water to give a final volume of 20 μL. An initial denaturation at 98°C for 2 min was followed by 50 cycles at 98°C for 5 s, 60°C for 5 s, and a melt curve 65°C–95°C on a Bio-Rad CFX96. Positive control mixtures, using DNA from *F. tularensis holarctica* and negative control mixtures without a template, were included in each PCR run.

Sequencing

The *lpnA* gene and FtM19InDel amplicons were cloned with TOPO TA cloning kit PCR4 (Invitrogen, Carlsbad, CA, USA) and sequenced. The acquired sequences were deposited in GenBank under accession nos. GY97987–GU97997 and HQ289871–HQ289876 (*lpn*A and FtM19InDel, respectively).

Results

F. tularensis in Mosquitoes Hatched from Field-collected Larvae

The 334 adult mosquitoes of 9 species hatched from mosquito larvae collected in the tularemia-endemic Örebro area were analyzed in 48 pools; 14 pools (29%) were positive for the *F. tularensis lpnA* gene (Table 1). Eleven of the 14 *lpnA*-positive samples were possible to sequence (Figure 1). All obtained sequences showed high sequence

Table 1. Presence of *Francisella tularensis lpnA* and *F. tularensis holarctica*–specific 30-bp–deletion region (FtM19InDel) in female and male mosquitoes, Örebro area, Sweden*

		Female mosquitoes			Male mosquitoes		
Group	Species	Pools	*F. tularensis*	*F. tularensis holarctica*	Pools	*F. tularensis*	*F. tularensis holarctica*
2a	*Aedes communis*	5 (10)	1	ND	–	–	–
2a	*Ae. intrudens*	2 (2)	ND	ND	–	–	–
2a	*Ae. punctor*	5 (5)	1	1	–	–	–
2a	*Aedes* spp.†	–	–	–	10 (31)	2	ND
2b	*Ae. cinereus*	7 (135)	3	ND	7 (109)	2	ND
2b	*Ae. sticticus*	4 (17)	2	ND	2 (12)	1	1
2b	*Ae. vexans*	2 (2)	1	1	–	–	–
1c	*Culiseta alaskaensis*	1 (1)	ND	ND	–	–	–
1c	*Cs. annulata*	1 (1)	ND	ND	–	–	–
1d	*Culex pipiens/torrentium*	1 (5)	ND	ND	1 (4)	1	1
Total		28 (178)	8	2	20 (156)	6	2

*Mosquitoes were collected as larvae in the Örebro area, central Sweden, reared to adults, and analyzed by real-time PCR in pools of up to 50 specimens. Numbers in parenthesis refer to total specimens within pools. ND, not detected; –, not analyzed.
†Male mosquitoes identified as *Aedes* spp. could belong to any species within functional group 2a and were not identified further. Mosquito functional groups are as defined by Schäfer et al. (*15*).

similarity (>97%) with *F. tularensis* in alignment with published sequences from representatives of subspecies of *F. tularensis* and their closest known relatives (i.e., *Francisella*-like endosymbionts).

We observed no difference in the *F. tularensis* detection rate between male (6/20 pools, 30%) and female (8/28 pools, 29%) mosquitoes (Table 1). Using the definitions in Schäfer et al. (*15*), we determined that the mosquito species belonged to 4 of 10 defined mosquito functional groups. Functional group 2a (snow-pool mosquitoes) and functional group 2b (floodwater mosquitoes) constitute most of the mosquitoes tested. Notably, the *F. tularensis* detection rate in female floodwater mosquitoes (6/13 pools, 46%) was higher than in female snow-pool mosquitoes (2/12 pools, 17%).

F. tularensis in Water

Water samples from 5 of the 8 water containers used for rearing were positive for the *F. tularensis lpnA* gene (Table 2). Four of these containers yielded mosquitoes that were positive for the *F. tularensis lpnA* gene. However, 3 water containers used for rearing that were negative for the *F. tularensis lpnA* gene, all yielded adult mosquitoes positive for the *F. tularensis lpnA* gene. Thus, there was no correlation between detection of *F. tularensis* in water from a specific container used for rearing and detection of *F. tularensis* in adult mosquitoes hatched from the container (Table 2).

Detection of *Francisella tularenis* spp. *holarctica*

Using the FtM19InDel primers, we detected sequences specific for *F. tularensis holarctica* in 2 of the 8 water samples analyzed and 4 of the 48 mosquito pools (Figure 2; Table 2). Mosquito species positive for *F. tularensis holarctica* sequences were *Ae. punctor* (snow-pool mosquito), *Ae. vexans*, *Ae. sticticus* (both floodwater mosquitoes), and *Culex pipens*/*torrentium* (Table 1).

Discussion

We detected *F. tularensis holarctica* DNA in adult mosquitoes hatched from field-collected larvae sampled in an area in Sweden endemic for tularemia. This finding suggests that mosquitoes came in contact with the causative agent of the disease, *F. tularensis holarctica*, in the aquatic habitat of the mosquito larvae. We have previously shown that *F. tularensis holarctica* persists in natural aquatic environments between outbreaks (*14*) and in association with protozoa (*10*,*11*). Mosquito larvae of floodwater mosquitoes (i.e., *A. sticticus*) are predators on protozoa in temporary wetland environments (*12*). Our results suggest natural transstadial transmission of *F. tularensis holarctica* from its water reservoir via female mosquitoes to their vertebrate blood-meal hosts, including humans. The observation of water containers negative for *F. tularensis* that yielded mosquitoes positive for the bacterium and vice versa suggests that mosquitoes were truly positive for *F. tularensis* and not cross-contaminated with water that tested positive; however, we cannot exclude varying sensitivity of the real-time PCR analysis for water and mosquito samples. Further studies of the tissue tropism of the bacterium within the mosquito body are needed to confirm how *F. tularensis holarctica* is transmitted by mosquito vector.

Transstadial transmission by mosquitoes after ingestion of pathogenic microorganisms as larvae has previously been shown for Rift Valley fever virus (*16*). The virus was transstadially transmitted to emerging adult mosquitoes (laboratory strain of *Cx. pipiens* and natural strains of *Ae. circumluteolus* and *Abydosaurus mcintoshi* from Kenya) that were capable of transmitting Rift Valley

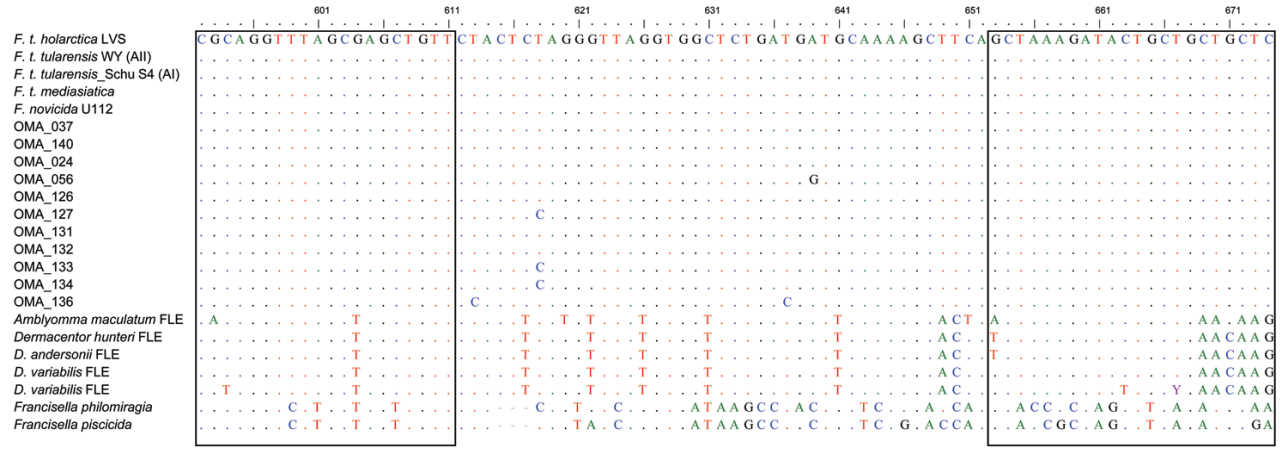

Figure 1. Multiple alignment of the 11 *Francisella lpnA* sequences obtained from mosquitoes in Sweden (hatched from field-collected larvae) with previously published sequences of *Francisella* species and subspecies, and *Francisella*-like endosymbionts (FLE). Boxed nucleotides represent target sequences of *lpnA* primers. The nucleotide positions 592–674 refer to *F. tularensis holarctica* live vaccine strain (LVS). Colors indicate individual nucleotides to clearly delineate those diverging from the *F. tularensis holarctica* LVS sequence. Reference sequences from GenBank; *F. tularensis holarctica* LVS (M32059), *F. tularensis tularensis* strain WY96-3418 (CP000608), *F. tularensis tularensis* strain Schu S4 (NC_006570), *F. tularensis mediasiatica* strain FSC147 (NC_010677), *F. tularensis novicida* strain U112 (CP000439), *Amblyomma maculatum* FLE (AY375422), *Dermacentor hunteri* FLE (AY375417), *D. andersonii* FLE (AY375413), *D. variabilis* FLE (AY375420), *D. variabilis* FLE (AY375421), *F. philomiragia* (AY243030), and *F. piscicida* (DQ825765).

fever virus to hamsters (*16*). Transstadial transmission of *F. tularensis* subspecies has also been reported in several species of ticks (*3*,*17*).

In a recent study, transmission of *F. tularensis novicida* was tested in laboratory strains of the tropical mosquitoes *Anopheles gambiae* and *Ae. aegypti* (*18*). However, the bacterium was not transmitted transstadially to adult mosquitoes, and female mosquitoes exposed to *F. tularensis novicida* in a blood meal were not able to transmit the bacterium to mice. Results of this study, along with our results, contribute to the growing body of data that indicate differences in the ecology, including vectors and reservoirs, of *Francisella* species, subspecies, and even populations (*3*,*14*,*19*,*20*).

We detected *F. tularensis* DNA in 29% of the pooled samples of adult mosquitoes hatched from field-collected larvae, indicating that transmission of the bacterium from water can generate a relatively high proportion of infected adult mosquitoes in an area endemic for tularemia. In line with our results, the *F. tularensis fopA* gene was detected in 30% of mosquito pools sampled in Alaska (*18*). However, further studies of host-seeking female mosquitoes in areas where tularemia is endemic are required to identify the range of mosquito species naturally infected with *F. tularensis holarctica* and the temporal distribution of the bacterium in these potential vector species in relation to the onset of outbreaks.

We detected *F. tularensis holarctica* in the floodwater mosquito species *Ae. sticticus* and *Ae. vexans,* the snow-pool mosquito species *Ae. punctor*, and a mixture of *Cx. pipiens* and *Cx. torrentium* mosquitoes. The 3 *Aedes* spp. mosquitoes feed primarily on mammals and commonly

Table 2. Presence of *Francisella tularensis lpnA* and *F. tularensis holarctica*–specific 30-bp–deletion region in water samples and in pools of mosquitoes hatched from larvae collected in the Orebro area, Sweden*

	Water			Mosquitoes		
Pond	Container	*F. tularensis*	*F. tularensis holarctica*	Pools	*F. tularensis*	*F. tularensis holarctica*
Ormesta 1	A	Yes	Yes	2 (2)	1	ND
	B	Yes	ND	6 (28)	ND	ND
Ormesta 2	C	ND	ND	6 (74)	2	ND
	D	Yes	ND	9 (79)	4	3
	E	ND	ND	6 (45)	3	ND
	F	Yes	ND	8 (61)	2	ND
	G	ND	ND	5 (27)	1	ND
Vattenpark	H	Yes	Yes	6 (18)	1	1
Total	8	5	2	48 (334)	14	4

*Water samples were collected from each mosquito-breeding container on arrival at the laboratory. Numbers in parentheses refer to number of mosquito specimens within pools. Sampling locations are Ormesta and Vattenpark. ND, not detected.

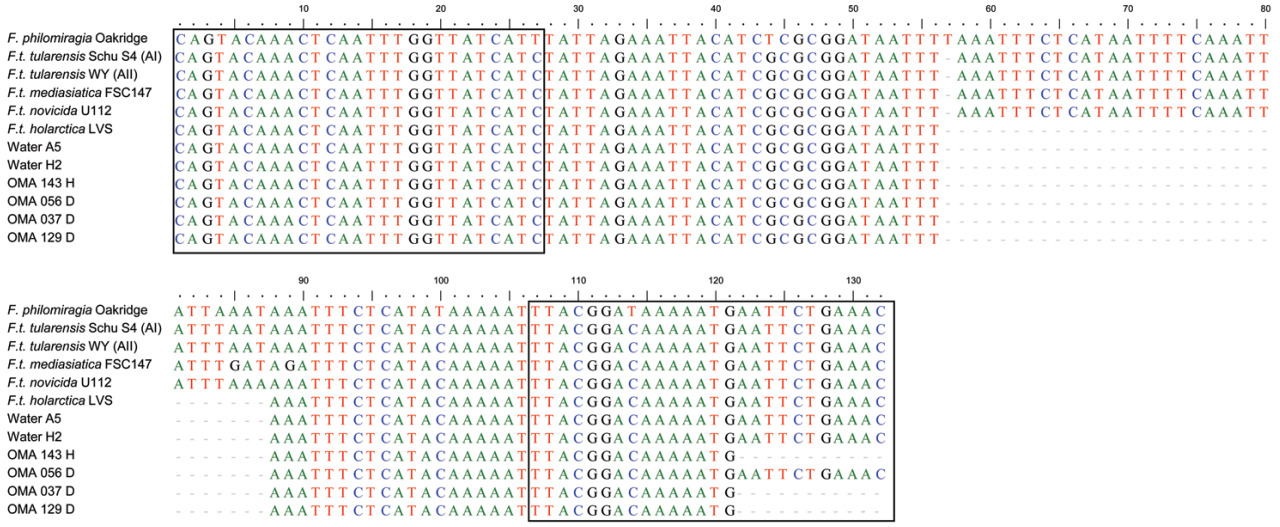

Figure 2. Multiple alignments of the 11 *Francisella lpnA* sequences (designated OMA_xxx) obtained from mosquitoes and water samples with previously published sequences of *Francisella* species and subspecies. Boxed nucleotides represent target sequences of FtM19InDel primers. In this alignment the *F. tularensis* subsp. *holarctica* specific deletion is located from position 57 to 87. Colors indicate individual nucleotides. Reference sequences from GenBank: *F. tularensis holarctica* LVS(M32059), *F. tularensis* subsp. *tularensis* strain WY96-3418 (CP000608), *F. tularensis* subsp. *tularensis* strain Schu S4 (NC_006570), *F. tularensis mediasiatica* strain FSC147 (NC_010677), *F. tularensis novicida* strain U112 (CP000439), and *F. philomiragia* (AY243030).

take blood meals from humans; the 2 *Culex* spp. mosquitoes feed on birds (*15*). With respect to blood-feeding habits, and the detection of *F. tularensis holarctica* DNA, all 3 *Aedes* spp. mosquitoes are potential vectors for transmission of *F. tularensis holarctica* to humans. Human cases of tularemia in Sweden (ulceroglandular and glandular) occur mainly in late summer and fall (*13*), a period when floodwater mosquito species are dominating the Swedish mosquito fauna (*21*). Notably, the detection rate of *F. tularensis* was higher in floodwater pools of female mosquitoes (46%) than in snow-pool pools of female mosquitoes (17%). The observation that *F. tularensis holarctica* occur in the floodwater mosquito *Ae. sticticus* is especially noteworthy because this nuisance species is now increasing its geographic range within Sweden (*22*).

We suggest that the transmission of the bacterium *F. tularenis holarctica* via blood-feeding mosquitoes to humans in areas of Sweden where tularemia is endemic originates from the aquatic habitat of mosquito larvae. However, further studies are needed to confirm transmission of the bacterium from its aquatic reservoir by blood-feeding female mosquitoes to their vertebrate hosts. The finding of *F. tularensis holarctica* DNA in adult mosquitoes, hatched from larvae collected in an area where tularemia is endemic, indicates that disease transmission in outbreaks originates in the pond habitats of the mosquito larvae.

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etymologia

Francisella tularensis

[fran-sĭ-sel′ə too″lə-ren-sis]

While studying plague in ground squirrels in 1911, George McCoy and Charles Chapin discovered a bacterium that caused a different disease. They named the pathogen *Bacterium tularense* after Tulare County, California, location of their study. In 1928, Edward Francis, a US Public Health Service bacteriologist, linked *B. tularense* with deer fly fever—tularemia transmitted by deer flies from infected wild rabbits to humans. In 1974, *B. tularense* was renamed *Francisella tularensis* in recognition of Dr. Francis' many contributions to our knowledge of tularemia.

Sources: Barry, J. Notable contributions to medical research by public health scientists. Public Health Service Publication No. 752. 1960 [cited 2011 Feb 25]. http://history.nih.gov/research/downloads/ Notable_Cont_Med_Research.pdf; Francis E. Sources of infection and seasonal incidence of tularemia in man. Public Health Rep. 1937;52:103–13; McCoy GW, Chapin CW. Further observations on a plague-like disease of rodents with a preliminary note on the causative agent, *Bacterium tularensis*. J Infect Dis. 1912;10:61–72; Sjöstedt A. Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations. Ann N Y Acad Sci. 2007;1105:1–29. doi:10.1196/annals.1409.009

Molecular Epidemiology of Oropouche Virus, Brazil

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Oropouche virus (OROV) is the causative agent of Oropouche fever, an urban febrile arboviral disease widespread in South America, with >30 epidemics reported in Brazil and other Latin American countries during 1960–2009. To describe the molecular epidemiology of OROV, we analyzed the entire N gene sequences (small RNA) of 66 strains and 35 partial Gn (medium RNA) and large RNA gene sequences. Distinct patterns of OROV strain clustered according to N, Gn, and large gene sequences, which suggests that each RNA segment had a different evolutionary history and that the classification in genotypes must consider the genetic information for all genetic segments. Finally, time-scale analysis based on the N gene showed that OROV emerged in Brazil ≈223 years ago and that genotype I (based on N gene data) was responsible for the emergence of all other genotypes and for virus dispersal.

Oropouche virus (OROV) is one of the most common orthobunyaviruses (family *Bunyaviridae*, genus *Orthobunyavirus*) (*1*) and is the causative agent of Oropouche fever in humans, which is clinically characterized as an acute febrile disease (*2*). The first isolation of OROV was reported in Trinidad and Tobago in 1955, when the virus was isolated from the blood of a febrile patient and from a pool of *Coquillettidia venezuelensis* mosquitoes (*3*). OROV was described in Brazil in 1960, when it was isolated from a sloth (*Bradypus tridactylus*) captured near a forested area during construction of the Belem–Brasilia highway and from a pool of *Ochlerotatus* (*Ochlerotatus*) *serratus* mosquitoes, captured near the same site (*4*).

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Since the first isolation of OROV, >30 outbreaks have been reported in Brazil, Peru, Panama, and Trinidad and Tobago during 1960–2009. At least half a million persons are estimated to have been infected (*5*,*6*).

Similar to the genomes of other orthobunyaviruses, the OROV genome comprises 3 single-stranded negative-sense RNA segments—large, medium, and small. The large RNA segment encodes a large protein that has RNA polymerase activity for transcription and replication of genomic RNA segments. The medium segment encodes a precursor polyprotein, which gives rise to the viral surface glycoproteins (Gc and Gn) and to a nonstructural protein NS_M. The small RNA encodes a structural nucleocapsid (N) protein, as well as a smaller nonstructural protein (NS_S) in overlapping reading frames (*1*). Studies of the molecular biology of the OROV small RNA segment have suggested its monophyletic origin and the existence of at least 3 genotypes (I, II, and III) (*7*). Recently, genotype III was isolated from a wild vertebrate host (*Callithrix* sp.) in southeastern Brazil, suggesting possible dispersion of the virus to susceptible and populated areas in Brazil (*8*). Further molecular analyses that used OROV strains recovered during outbreaks in Pará State during 2003–2007 demonstrated the association of at least 2 different genotypes (I and II) with Oropouche fever cases in the area (*9*,*10*).

In this study, we describe new information regarding the molecular epidemiology of OROV. This information will help clarify the evolution, dispersal, and genotyping classification of this human pathogen in the Brazilian Amazon region.

Material and Methods

Virus Strains

The OROV strains used in this study (online Appendix Table, www.cdc.gov/EID/content/17/5/800-appT.htm)

were relatively low-passage isolates obtained from the virus collection of the Department of Arbovirology and Hemorrhagic Fevers, Evandro Chagas Institute (Ananindeua, Brazil). These strains corresponded to viruses recovered from different hosts and geographic locations that were isolated during 1960–2009.

Virus Culture and RNA Extraction

Viruses were propagated in monolayer cultures of Vero cells. After 75% of cells showed cytopathic effects, the supernatants of infected cell cultures were collected. RNA extraction was conducted by using a commercial kit (QIAmp Viral RNA Mini Kit; QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions.

Reverse Transcription–PCR and Nucleotide Sequencing

For the synthesis and amplification of the OROV small RNA, medium RNA, and large RNA DNA (cDNA), a 1-step reverse transcription–PCR (RT-PCR) was conducted by using a combination of specific-segment sets of the following primers: small RNA (NORO5: AAAGAGGATCCAATAATGTCAGAGTTCATTT; ORO N3: GTGAATTCCACTATATGCCAATTCCGAA TT), medium RNA (Gn15S: GGCAACAAACAGTGA CAAT and Gn659R: CTATGTTAACGCACATTGCT), and large RNA (LOROF: CCGAAACAAACAAAAACAAT; and large RNA (LOROF: CCGAAACAAACAAAAACAAT and LOROR: GGATGAGTAAGCAATTCTGG) (*7*). Amplicon lengths were expected to be 693 bp, 644 bp, and 634 bp for small RNA, medium RNA, and large RNA, respectively. The RT-PCR products were visualized onto 1.2% agarose gel stained with ethidium bromide (0.5 μg/mL). Amplicons were sequenced by using the same primers applied for the RT-PCR cycling and the ABI PRISM Dye Terminator Kit (Applied Biosystems, Foster City, CA, USA) by using the dideoxyribonucleotide chain terminator method (*11*). The ABI 3130 capillary automated sequencer (Applied Biosystems) was used to obtain the sequence. Both cDNA strands were sequenced from at least 3 RT-PCR products.

Sequence Analysis and Phylogeny

Sequences obtained for the N (complete), Gn, and large (partial) genes were first inspected in quality by the SeqMan LaserGene package (DNA STAR, Madison, WI, USA) and then used for multiple sequencing alignments with other OROV sequences available in GenBank (www.ncbi.nlm.nih.gov/genbank). The genetic divergence for each gene was determined by using MEGA4 software (*12*) based on the dataset generated by the alignments. Confidence interval for inclusion into a given phylogenetic group was estimated according to the mean of genetic divergence calculated for the known OROV genotypes (I, II, and III) and used as a criterion for searching other genotype groups.

The phylogenetic analysis was performed by comparing the 66 entire N genes and 36 partial Gn genes and large sequences of Brazilian OROV strains, respectively, with homologous sequences obtained from other OROVs isolated from different regions of Central and South America, periods of time, and source of isolation (Table). Phylogenetic trees were constructed by using the neighbor-joining (*13*), maximum-likelihood, and maximum-parsimony methods in the PAUP 4.0 software (*14*) as described (*8*). Bayesian and time-scaled (chronologic) analyses also were conducted as described by Rodrigues et al. (*15*). Sequences obtained from the OROV isolates were deposited in GenBank (Table, GenBank accession numbers of previously sequenced OROV and other Simbu group virus strains; online Appendix Table).

Evaluation of RNA Segment Topologies

To evaluate the topologies presented by the different RNA segments, we used 36 OROV strains for which all 3 segments were sequenced. The evaluation was performed by using the Kishino-Hasegawa method (*16*), comparing a topology generated for a given RNA segment with the other segments. We considered p values ≤ 0.01 significant.

Results

Genetic Variability of OROV

The nucleotide sequences obtained for the studied strains were 693 nt (231 aa), 644 nt (214 aa), and 634 nt (211 aa) in length for N, Gn, and large genes, respectively. The multiple sequencing analysis of the new 66 full-length OROV N (small RNA) and for the 36 partial Gn (medium RNA) and large RNA gene sequences showed high nucleotide and amino acid identities (>90%). The mean of genetic divergence among the N gene nucleotide sequence was ≈6.8%. Genetic distances (nucleotide sequence) within the 3 well-established genotypes (I, II, and III) ranged from 3% between genotypes I and II to 4.4% between genotypes I and III (mean 3.5%) and were used as a confidence value for inclusion within a given genotype. On the basis of this criterion, a fourth group was established, and a genetic divergence ranging from 5.3% with genotype I to 6.8% with genotype III (mean 5.8%) was determined. The mean of genetic divergence among the 4 OROV lineages was 4.6% (Table).

Regarding the Gn gene nucleotide sequences, the analysis showed values of genetic divergence of 0.9%–9.5% (mean 6.5%). In contrast to the N gene sequences, for the Gn gene partial sequences, 3 lineages were identified, showing an intergroup divergence of 4.5% (between groups

Table. Percentage of genetic divergence between Oropouche virus phylogenetic groups on the basis of the complete N (small RNA) and partial Gn (medium RNA) and L (large RNA) gene sequences, Brazil*

Gene and group	Divergence among genotypes,%				Mean of genetic divergence intergroup
	I	II	III	IV	
N gene					
I					
II	3.0 (2.0)				
III	4.4 (3.1)	3.0 (2.0)			
IV	5.3 (3.6)	5.3 (3.6)	6.8 (3.9)		
IV in relation to I, II, and III					5.8 (4.0)
I, II, and III					3.5 (2.3)
I, II, III, and IV					4.6 (3.4)
Gn gene					
I					
II	4.5				
III	7.2	5.6 (3.8)			
III in relation to I and II					5.7 (3.8)
L gene					
I					
II	0.8 (0)				
II in relation to I					0.5 (0)

*Percentages within parenthesis are the amino acid sequence divergences among Oropouche virus strains.

I and II) to 7.2% (between groups I and III) (mean 5.7%), which was used as a confidence value for group inclusion or exclusion (Table).

For the polymerase gene nucleotide sequences (large RNA), genetic divergence was 0.1%–0.8% (mean 0.5%). Only 2 large RNA segments were distinguished into groups (Table).

Phylogeny and Time-scaled Analysis

Regardless of the method used, the trees were similar in topology, showing high support values (bootstrap, likelihood, or posterior probability values). The Bayesian method showed high support values (>0.90) and was therefore used to represent the final tree. As previously reported (*7–10*), the comparative phylogeny that used the entire N gene sequences (96 strains; online Appendix Table) confirmed the monophyletic origin of OROV in comparison with other Simbu group viruses (Figure 1),

The 4 major phylogenetic groups depicted (I–IV) corresponded to 4 distinct genotypes (Figure 2 [Bayesian method]). Genotype I included the Brazilian strains isolated in the states of Acre, Amazonas, Maranhão, Tocantins, and Pará, as well as strains from Trinidad and Tobago. Three subgenotypes were described: Ia, Ib, and Ic (Figure 1). Genotype II grouped strains obtained during outbreaks in the states of Amapá, Pará, and Rondônia in Brazil and the strains from Peru. Three subgenotypes also were assigned to this group (II a, II b, and II c). Genotype III was formed by strains isolated in the Brazilian states of Acre, Minas Gerais, and Rondônia, and the isolates from Panama showing 2 distinct sublineages: the subgenotypes II a and III b. Finally, genotype IV included the Brazilian strains isolated in Amazonas State, Brazil (Figure 2).

Chronologic analysis was used to investigate the emergence period of OROV in the Americas. The nucleotide substitution rate that determined the 96 OROV N gene sequences was 3.7×10^{-4} substitutions per site per year and was used to estimate the divergence dates among the strains. The emergence of the most recent common ancestor (MRCA) for OROV in the Americas was estimated to have occurred ≈223 years ago (95% highest probability density [HPD] 148–342 years) from the location where the other parental viruses for the different genotypes (I, II, III, and IV) emerged (Figure 2). The estimated emergence dates suggest that genotype I was the first genotype that emerged ≈112 years ago (95% HPD 95–189 years). Genotype II emerged ≈91 years ago (95% HPD 59–144 years) and originated from strains isolated in the states of Pará and Rondônia, and strains recently isolated in Amapá State, in 2009. Genotype III was estimated to have originated 37 years ago (95% HPD 33–70 years) and probably evolved in Rondônia State 33 years ago (95% HPD 29–58 years), and other Amazonian states, such as Acre and Pará, emerging almost simultaneously in Panama 32 years ago (95% HPD 22–45 years) and, more recently, in Minas Gerais State. Genotype IV emerged in Amazonas State ≈43 years ago (95% HPD 31–56 years; Figure 2).

Evaluation of RNA Segment Topologies

Trees generated from entire N and partial Gn and large gene sequences obtained for 36 OROV strains demonstrated different topologies. By using all phylogenetic methods, we found differences in virus clustering. For the small RNA, 4 distinct groups were identified: group I (20 strains), group II (9 strains), group III (5 strains), and group IV (2 strains). For the medium RNA, 3 groups were assigned and distributed

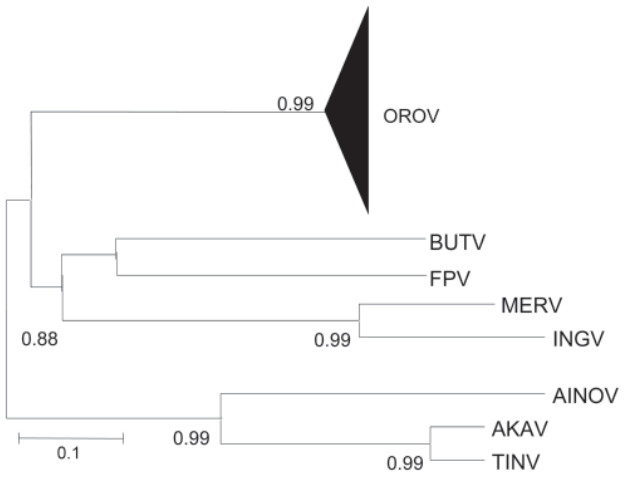

Figure 1. Phylogenetic analysis between Oropouche virus (OROV) (N gene: 693 nt) and homologue sequences of different viruses that belong to the Simbu group. AINOV, Aino virus; AKAV, Akabane virus; TINV, Tinaroo virus; BUTV, Buttonwillow virus; FPV, Facey's Paddock virus; MERV, Mermet virus; INGV, Ingwavuma virus. The numbers above each main node correspond to bootstrap values for phylogenetic groups. Scale bar indicates 10% genetic divergence.

as follows: group I (28 strains), group II (4 strains), and group III (4 strains). The large RNA depicted only 2 major groups, including 32 strains in group I and 4 strains in group II (Figure 3). Maximum likelihood was used to analyze these competing small, medium, and large segment topologies by using the Kishino-Hasegawa test. Sequence evolution models were optimized by applying all genome segments and using the competing topologies. Regardless of which model was selected, each topology generated by using maximum parsimony and neighbor-joining methods with a given genome segment was significantly more likely than the competing topology generated by using the other genome segment (likelihood probability between S and M topologies = 0.00005623; likelihood probability between S and L topologies = 0.000354664; likelihood probability between M and L topologies = 0.00043154; $p<0.001$).

Geographic Dispersion of OROV Genotypes

On the basis of results obtained for the N gene data by time-scaled analysis (evolutionary rate and emergence date) and epidemiologic data association (date and place of isolation), the possible dispersal event could be predicted for the distinct OROV genotypes in the Americas (Figure 4). Genotype I (dispersion route in red), originally isolated in Brazil in the municipality of Ipixuna, Pará State (BR 010 Highway, km 94), possibly dispersed continuously toward distinct directions: initially to several municipalities in western Pará, and simultaneously in Trinidad and Tobago. Later, genotype I moved toward the states of Amazonas and Acre and, more recently, to the eastern Amazon region including Pará, Maranhão, and Tocantins States. Genotype II (dispersion route in dark blue), apparently emerged simultaneously in the states of Amapá, Pará, and Rondônia, as well as in Peru, and dispersed in these places, emerging in the municipality of Mazagão, Amapá State, in 2009. Genotype III (dispersion route in green), emerged in Rondônia State, moving toward Panama and the states of Acre and Maranhão. From Maranhão, a new route led genotype III to the Minas Gerais State. Genotype IV (black dot in Manaus), apparently more ancient than genotype III, emerged in the city of Manaus, Amazonas State, and it has not apparently dispersed from there (Figure 4).

Discussion

The molecular epidemiology of OROV has been extensively studied on the basis of genetic data generated for the small RNA segment, and the data have provided information about the genetic diversity of OROV and geographic distribution in countries in which the virus is endemic, such as Brazil, Peru, and Trinidad and Tobago (*7–10*,*17*). The analysis of additional 66 gene sequences of the entire N and partial Gn and Gc provided a better understanding of the molecular epidemiology of OROV in Brazil. In our analysis, distinct phylogenetic groups

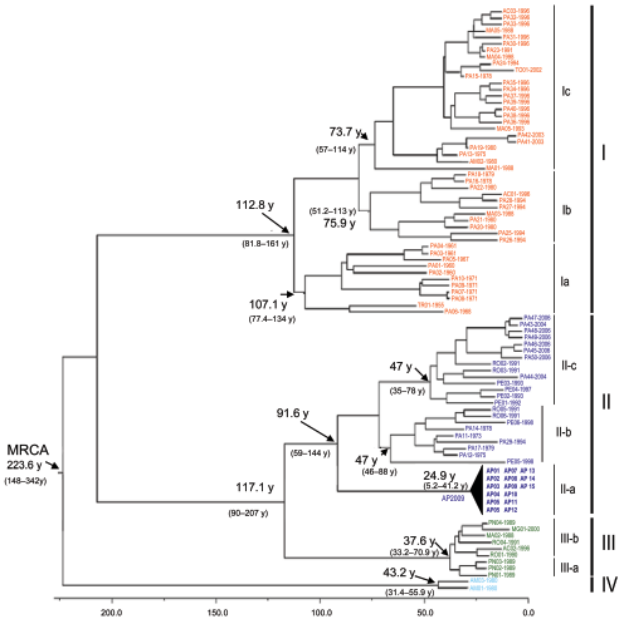

Figure 2. Phylogenetic tree based on the complete nucleotide (nt) sequence of the N gene (693 nt) of 96 Oropouche virus (OROV) strains isolated from different hosts, locations, and periods. The main phylogenetic groups are represented by genotypes I (red), II (dark blue), III (green), and IV (light blue). The values above the main nodes represent the dates of emergence of common ancestors, expressed in years before 2009. The arrows indicate the probable date of emergence of genotypes I, II, III, and IV. Numbers in parentheses are value for 95% highest probability density. Scale bar indicates time scale of molecular dating. MRCA, most recent common ancestor.

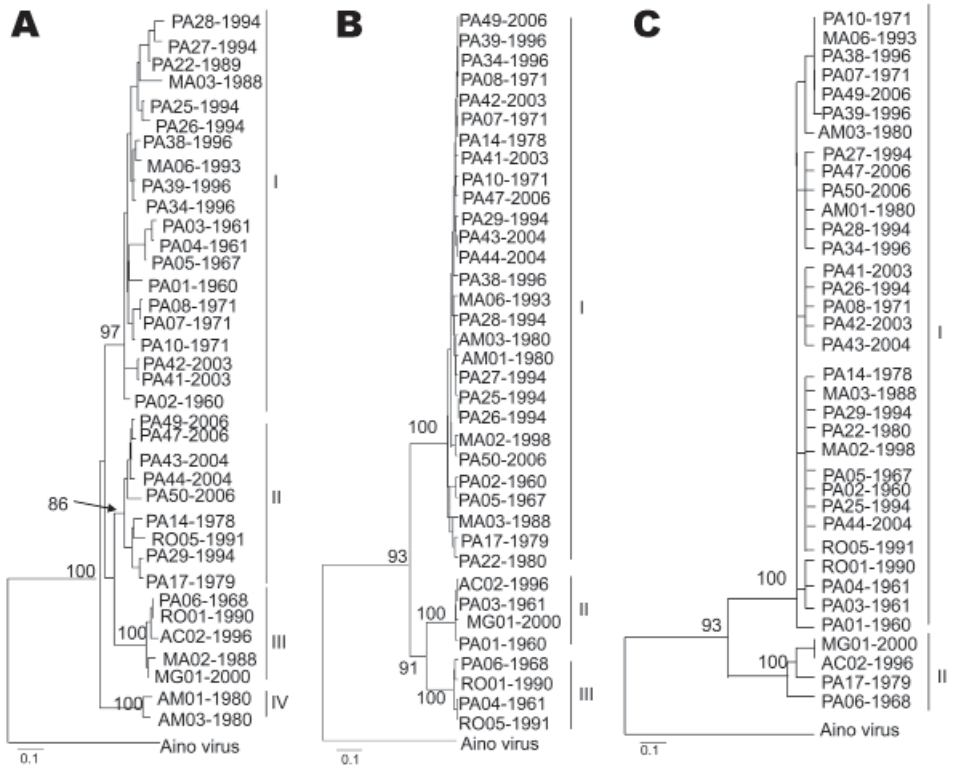

Figure 3. Phylogenetic analysis of 36 Oropouche virus strains: A) N gene (693 nt), B) Gn gene (644 nt), and C) large (L) gene (634 nt), showing different topologies. Bootstrap values obtained by using maximum parsimony and neighbor-joining methods are placed over each main node of the tree corresponding to the phylogenetic groupings. The arrow indicates the exact position of the bootstrap value in the tree. Scale bars indicate 10% nt divergence.

were observed when the different RNA segments were analyzed. In case of the small RNA, 4 major groups were found, including the 3 genotypes previously described (*7–10*,*17*). Although a fourth genetic lineage has been well established by the small RNA phylogeny (strains AM 01 and AM 03), the topologies depicted by the medium RNA and large RNA sequences did not support this result. Maximum likelihood analyses were used to test these competing small, medium, and large segment topologies by using the Kishino-Hasegawa test. Evolution models were optimized for all 3 genome segment sequences and by using the competing topologies. Regardless of which model was selected, each topology generated by using maximum parsimony and neighbor-joining methods with a given RNA segment was significantly more likely than the competing topology generated by the other genome segment ($p<0.001$) (Figure 3). These results ensured that the testing topologies obtained for each RNA segment differed significantly, which suggests that each OROV RNA segment had a different evolutionary history and probably contributes to the genetic variability of the virus.

The assessment of additional genetic data for the small RNA segment contributed substantially to the understanding of the emergence of the virus, geographic distribution, and dispersal events. On the basis of chronologic dating of the N gene, epidemiologic data, and lineage definition (genotypes I–IV), we were able to elucidate the possible origin of OROV in the Americas (Figures 2, 4). In contrast to information about the event in Trinidad and Tobago in 1955 that was associated with the first description of the Oropouche fever case, molecular data provided by the small RNA sequences indicated that OROV emerged in South America, more precisely in Pará State (strains PA 01–PA 05) in northern Brazil, ≈89 years ago, and then in Trinidad and Tobago probably through humans carrying the virus during the viremic phase or through illegal shipment of wild animals, as has been suggested for yellow fever virus (*18*).

The dispersal history of OROV strains is initially associated with genotype I, more precisely with the subgenotype Ia, isolated from wild animals and humans during epidemics in Pará State, during the 1960s–1970s. Their dispersion routes were simultaneously west to east in the Amazon toward Acre State (subgenotype Ib) from 1988 to 1994 and, more recently, in a vast area in Pará State and in Manaus, Amazonas State, at the end of the 1990s and the beginning of the 2000s.

Regarding genotype II, the most probable origins were in eastern Pará (Porto de Moz) toward Iquitos, Peru (subgenotype IIb), and from Iquitos toward Ariquemes, Rondônia State (subgenotype IIc), where the virus probably then dispersed to Madre de Dios in Peru and to Pará State. The origin of subgenotype IIa, which is represented by the strains recently associated with the epidemic in Mazagão,

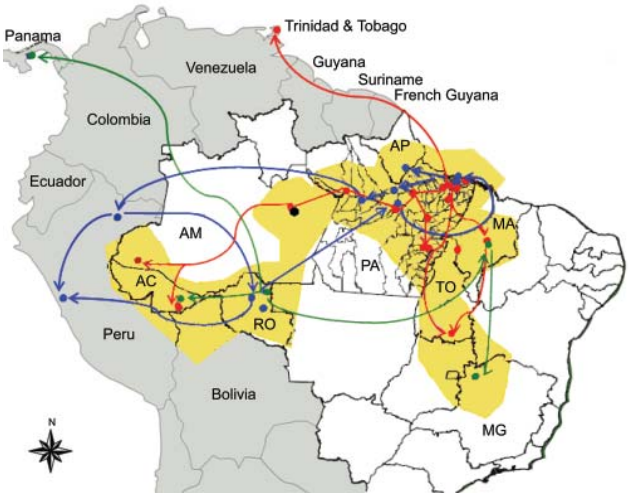

Figure 4. Geographic dispersion of Oropouche virus (OROV) genotypes in South America during 1955–2009 based on data from the N gene. Yellow shading, coverage area of OROV in Brazil; red line, dispersion route for genotype I; blue line, dispersion route for genotype II; green line, dispersion route for genotype III; black dot, genotype IV. AC, Acre; AP, Amapá; AM, Amazonas; MA, Maranhão; MG, Minas Gerais; PA, Pará; RO, Rondônia, TO, Tocantins.

Amapá State, in the beginning of 2009 (P.F.C. Vasconcelos et al., pers. comm.) is probably related to a common ancestor that evolved independently from other subgenotypes (IIb and IIc) over time and probably emerged in the Amazon ≈24 years ago.

The existence of genetic data for a single genotype III Brazilian strain isolated in Minas Gerais State, southeastern Brazil (*8*), limited our ability to study its origin and evolutionary aspects. With the identification of other genotype III strains in Brazil, isolated in the states of Rondônia (Ariquemes and Machadinho d'Oeste), Acre (Xapuri), and Maranhão (Porto Franco), we were able to make inferences about the most possible dispersion route. In fact, it constitutes a complex dynamics of evolutionary origin between subgenotypes IIIa (predominantly from Brazil) and IIIb (predominantly from Panama). In this context, genotype III probably originated from the sublineage IIIa, which was isolated in Ariquemes, Rondônia State, from which the sublineage IIIb ancestor has segregated independently, leading to the emergence of strains in Chame and San Miguelito, Panama.

In a more detailed view, the subgenotype IIIa found in Ariquemes, Rondônia State, had its initial dispersion to a neighboring municipality (Machadinho d'Oeste), subsequently to Porto Franco in Maranhão State, and finally to Arinos, Minas Gerais State. Although Minas Gerais State is geographically distant from the official OROV-endemic area, the virus may have been introduced through Maranhão State by the intense traffic of humans from Maranhão to other states and regions in Brazil.

In Minas Gerais, OROV has been maintained in silent cycles, probably because of inadequate epidemiologic conditions, such as the high density of *Culicoides paraensis* mosquitoes in urban areas, a limiting factor for an epidemic cycle deflagration. Furthermore, the sporadic detection of OROV was recently reported in Acre State (*19*); these reports confirmed that the virus actually circulates silently in the Brazilian Amazon, as suggested by Azevedo et al. (*9*), and can be transported by viremic patients and human carriers of subclinical illness from region to region within the country. This approach should result in stronger data when new isolates are sequenced in other OROV-endemic countries because limited information about dispersal of OROV in Peru, Panama, and Trinidad and Tobago does not infer a more robust analysis.

In conclusion, even with the limited data obtained in this study from other OROV-endemic countries, we were able to reach a more complete understanding of the molecular epidemiology of the virus, and we provided evidence of which distinct genes (N, Gn/Gc, and L) are under different selective evolutionary pressures in nature. We also observed the great genetic diversity of OROV, the description of a new genotype IV, the complex dynamics of evolution, and viral dispersal. Finally, our findings suggest the necessity of obtaining genetic data regarding full-length sequencing of different OROV strains (medium and large segments) to elucidate the correct genotype classification and to improve the molecular diagnostics of this human pathogen in Latin America.

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Severe Imported *Plasmodium falciparum* Malaria, France, 1996–2003

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Release date: April 22, 2011; Expiration date: April 22, 2012

Learning Objectives

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

- Describe the diagnosis and prognosis of malaria among travelers
- Distinguish characteristics of returning travelers with malaria in the current study
- Analyze variables associated with a higher risk for severe malaria among returning travelers

Editor

Beverly Merritt, Technical Writer/Editor, *Emerging Infectious Diseases. Disclosure: Beverly Merritt has disclosed no relevant financial relationships.*

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Little is known about severe imported *Plasmodium falciparum* malaria in industrialized countries where the disease is not endemic because most studies have been case reports or have included <200 patients. To identify factors independently associated with the severity of *P. falciparum*, we conducted a retrospective study using surveillance data obtained from 21,888 *P. falciparum*

[1]Additional members of the French National Reference Center for Imported Malaria Study Group who contributed data are listed in the online Technical Appendix (www.cdc.gov/EID/content/15/5/807-Techapp.pdf).

patients in France during 1996–2003; 832 were classified as having severe malaria. The global case-fatality rate was 0.4% and the rate of severe malaria was ≈3.8%. Factors independently associated with severe imported *P. falciparum* malaria were older age, European origin, travel to eastern Africa, absence of chemoprophylaxis, initial visit to a general practitioner, time to diagnosis of 4 to 12 days, and diagnosis during the fall–winter season. Pretravel advice should take into account these factors and promote the use of antimalarial chemoprophylaxis for every traveler, with a particular focus on nonimmune travelers and elderly persons.

Each year, a growing number of persons from industrialized countries travel to developing countries. Among these millions of travelers, 20% to 70% report some illness associated with their travel and ≈3% report fever (*1*). Malaria appears to be the most common cause of fever in returned travelers. Because of its potential severity, *Plasmodium falciparum* infection must be considered in all febrile persons who return from an area where malaria is endemic. Depending on the country of importation and the year, ≈2%–16% of *P. falciparum* imported malaria infections are severe cases according to the World Health Organization (WHO) definition, and ≈10% of severe cases end with the death of the patient despite appropriate antimalarial treatment (*2*).

Globally, epidemiologic and pathophysiologic research studies on malaria are mainly based on disease in children <5 years of age in areas where malaria is endemic. Little is known about severe imported malaria, which primarily affects nonimmune adults. Most previous studies that have focused on severe imported malaria have been case reports or have included <200 patients (*3*–*12*). Recently, we published an analysis of risk factors associated with death in a series of 96 malaria-related deaths of 21,888 patients with imported *P. falciparum* malaria (*2*). Characteristics independently associated with death were older age, being a native of an area where malaria is nonendemic, infection occurring in eastern Africa, and absence of appropriate chemoprophylaxis. This database, including the characteristics of 832 case-patients with severe disease, provides a unique opportunity to analyze the risk factors for severe imported malaria in France. We conducted a retrospective analysis of the main features of severe imported malaria cases compared with nonsevere cases that occurred during 1996–2003 in France. Increased knowledge in this area may lead to improvement in terms of prevention and patient management.

Materials and Methods

Data Sources

Imported malaria is not a mandatory notifiable disease in metropolitan (mainland) France. Data for this study were collected by a reporting network of 120 selected hospital laboratories and were analyzed by the French National Reference Center for Imported and Autochthonous Malaria Epidemiology (CNREPIA). Participants of the network were asked to report imported malaria cases whenever asexual forms of *P. falciparum* were seen by laboratory observations of a patient's blood film. Data from the national medical informatics systems and from 2 exhaustive studies (National Quality Control Survey) suggested that these cases represented 50%–55% of the total number of imported *P. falciparum* malaria cases in France during the study period (*13,14*). A standard 57-item questionnaire, completed by clinicians and biologists for each reported case, collected basic demographic, epidemiologic, clinical, and parasitologic information (including prophylaxis and treatment).

Data Collection

The study population consisted of all *P. falciparum*–infected patients reported to CNREPIA during 1996–2003. We used WHO criteria for the definition of severe *P. falciparum* malaria as the primary outcome (*10,11*). The 1990 WHO definition (*15*) was used for cases occurring before 2000 (Table 1); the revised 2000 definition (*16*) was used for those occurring after 1999 (Table 2).

Table 1. Criteria used before 2000 to define severe malaria, 1990 World Health Organization definition

Major criteria
Unrousable coma
Glasgow Coma Scale score of ≤9
Repeated generalized seizures
Circulatory collapse, systolic blood pressure <80 mm Hg despite adequate volume repletion
Pulmonary edema with presence of criteria for acute respiratory distress syndrome or acute lung injury
Spontaneous bleeding and/or disseminated intravascular coagulation
Acidemia, pH <7.35, or acidosis, serum bicarbonate <15 mmol/L
Severe anemia, hemoglobin <5 g/dL
Renal impairment, serum creatinine >265 μmol/L
Hypoglycemia, blood glucose, <2.2 mmol/L
Macroscopic hemoglobinuria (if unequivocally related to malaria)
Minor criteria
Impaired consciousness but rousable
Extreme weakness
Temperature >40°C
Parasitemia >5%
Jaundice or total bilrubin >50 μmol/L

Table 2. Criteria for severe malaria, World Health Organization definition revised in 2000

Extreme weakness
Impaired consciousness, Glasgow Coma Scale score <9
Pulmonary edema with presence of criteria for acute respiratory distress syndrome or acute lung injury
Repeated generalized seizures, >2 within 24 h
Circulatory collapse
Systolic blood pressure <80 mm Hg despite adequate volume repletion
Spontaneous bleeding and/or disseminated intravascular coagulation
Jaundice or total bilirubin >50 μmol/L
Macroscopic hemoglobinuria (if unequivocally related to malaria)
Severe anemia
Hemoglobin <5 g/dL
Hypoglycemia, blood glucose <2.2 mmol/L
Acidemia, pH <7.35, or acidosis, serum bicarbonate <15 mmol/L
Hyperlactatemia, arterial lactate >5 mmol/L
Acute renal failure, urine output of <400 mL/24 h and serum creatinine >265 μmol/L
Parasitemia ≥4%

In the 1990 WHO definition, only major criteria were used to define severe malaria. In the revised WHO definition in 2000, major and minor criteria were grouped together (except temperature criteria) to expand the definition of severe malaria. In addition, 2 criteria were changed: acute renal failure with urine output of <400 mL/24 h and serum creatinine >265 μmol/L and >2 generalized seizures within 24 h.

The case severity rate per 100 patients was calculated for all relevant exposure variables. Various exposure categories created for the first study (*2*) were used in this analysis. Patients were divided into categories: European travelers (persons born and residing in a country in Europe not endemic for malaria); European expatriates (persons born in a country in Europe where malaria is not endemic and residing in a sub-Saharan African country where malaria is endemic); African travelers (persons born in a sub-Saharan African country where malaria is endemic and residing in France or another country where malaria is not endemic); African residents (persons born and residing in a sub-Saharan African country where malaria is endemic); and others. Use of malaria chemoprophylaxis was categorized as reported by patients: no use; use of inappropriate drugs (chloroquine, proguanil alone, pyrimethamine, and sulfadoxine-pyrimethamine); and use of appropriate drugs (mefloquine, atovaquone-proguanil, doxycycline, and chloroquine-proguanil) according to recommendations from the Haut Conseil de la Santé Publique (www.hcsp.fr).

Data Analysis

Logistic regression was used to identify factors associated with severe malaria and to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the association between exposure variables and severe cases. Dummy variables were used for variables with >2 categories. Variables with p values <0.25 were introduced in the multivariate logistic regression model. A manual backward stepwise approach was used to remove nonsignificant variables; only variables with p values <0.05 were retained in the final model. Interactions were sought by introducing interaction terms in the logistic regression model and testing for their significance at the 0.05 level. Moreover, because our data ranged from 1996 through 2003 and we used 2 WHO definitions for severe disease (*15,16*), we systematically tested interactions between year of diagnosis and all relevant associations; all analyses were adjusted for the year of diagnosis. Statistical analysis was performed by using Stata 10 (StataCorp LP, College Station, TX, USA).

Results

During the 1996–2003 study period, 27,085 malaria cases were reported to CNREPIA. Of these, 21,888 cases were *P. falciparum* malaria (the study population), among which 20,431 (93.3%) were uncomplicated, 832 (3.8%) were severe, 433 (2%) were asymptomatic, 160 (0.7%) showed hyperreactive malarial splenomegaly, and 32 (0.1%) were unspecified. Among the 21,888 *P. falciparum* cases, 96 deaths (case-fatality rate [CFR] of 0.4%) were related to malaria. The annual number of malaria cases reported by the network increased to ≈3,000 cases up to 1999–2000 and then incidence began to regularly decrease. The CFR of severe *P. falciparum* malaria was higher during 2000–2003 than before, reaching 15.3% in 2003 (Table 3).

Most patients were male (sex ratio M:F = 1.7), and the median age was 29.6 (range 0–96) years. African travelers were most numerous (44.6%), followed by European travelers (26.5%), African residents (12.9%), and European expatriates living in Africa (5.4%); "others" represented 10.6%. Most patients (97.5%) became infected with malaria in Africa: 59.2% in western Africa, 26.2% in central Africa, 11.2% in Madagascar and the Comoros Islands, and 0.9% in eastern Africa (Table 4; online Appendix Table). Others (2.5%) had returned mainly from French Guiana, Haiti, India, Sri Lanka, Thailand, and Indonesia. Almost one third of the patients (30.4%) stated they had taken an appropriate chemoprophylaxis, whereas more than half stated they had not. The median duration of stay was 32 days (interquartile range [IQR] 21–62 days). The median time from return to symptom onset was 6 days (IQR 1–12 days), and 11% of patients had their first symptom before returning to France. The median time from symptom to diagnosis was 3 days (IQR 1–6 days). Most patients went to a hospital first (69.4%) and, compared with Europeans, Africans were more likely to do so (73% vs. 62.7% p<0.001). At the time of diagnosis, 7.9% of patients had high-level parasitemia (>5% of parasitized erythrocytes). Seventy-five percent of

Table 3. Distribution of *Plasmodium falciparum* malaria cases and severe cases by calendar year, France, 1996–2003*

Year	*P. falciparum* malaria cases	No. (%) severe cases	Deaths	CFR for all cases, %	CFR for severe cases, %
1996	1,804	96 (5.3)	8	0.4	8.3
1997	2,057	94 (4.5)	10	0.5	11.0
1998	2,459	115 (4.7)	9	0.3	7.8
1999	3,385	118 (3.5)	9	0.2	7.6
2000†	3,355	84 (2.5)	12	0.3	14.3
2001	3,035	90 (3.0)	13	0.4	14.4
2002	2,919	105 (3.6)	15	0.5	14.3
2003	2,874	130 (4.5)	20	0.7	15.3
Total	21,888	832 (3.8)	96	0.4	11.3

*CFR, case-fatality rate.
†Year World Health Organization revised definition for *severe P. falciparum* malaria.

patients were hospitalized, and the median hospitalization length was 3 days (range 0–169 days). Laboratory results for 13.1% of patients revealed low platelet counts ($\leq 50 \times 10^9$ cells/L); 6% of patients had low hemoglobin levels ($\leq$8 g/dL).

Description of Severe Cases

A total of 832 patients had severe malaria according to WHO definition (51% recorded before the revision in 2000). From 1996 through 2003, the evolution of the number of severe cases was the inverse of that of the total number of imported *P. falciparum* cases. Since 2000, we observed a slow decrease in the total number of imported *P. falciparum* cases, whereas severe cases increased in number and proportion (Table 3).

Of the 832 patients who had severe malaria, 386 (46.4%) were European travelers, 98 (11.8%) European expatriates, 190 (22.8%) African travelers, 73 (8.8%) African residents, and 85 (10.2%) others. Sex ratio (M:F) was 2.3, and the median age was 38.2 years (range 0–92 years). Twenty-seven patients (3.2%) were >70 years of age, and 127 (15.3%) were <15 years of age. With a 15.3% CFR, eastern Africa accounted for the highest proportion of severe cases. Two-hundred-fourteen (27.9%) of these patients stated they had taken an appropriate chemoprophylaxis. Initial visit to a general practitioner instead of a hospital occurred for 351 (43.8%) patients. A total of 803 patients (98.5%) were hospitalized, 540 (66.2%) in an intensive care unit; the median hospitalization length was 6 days (range 0–169 days). The description of the 96 fatal cases has been reported elsewhere (*2*).

Factors Associated with Severe Cases

The CFR was 3.8% during the study period and changed over time (decrease from 1996 to 2000, then an increase since; $p<0.0001$ by standard χ^2 test or trends test). Many factors were associated with an increased risk of severe malaria in univariate analysis, including older age, male gender, European origin, infection in East Africa, short stays (≤21 days), inappropriate chemoprophylaxis, initial visit to a general practitioner, time to diagnosis, and diagnosis during the fall–winter season (online Appendix Table).

However, short stays and male gender were no longer predictive of severity after controlling for all the variables, including year of diagnosis. Table 5 shows the results of multivariate analysis. Independent factors associated with severe malaria were no different whether patients were African or European, and the year of diagnosis did not modify the effect of the associations.

Discussion

With a mean of >4,000 cases per year during the study period, France is the country reporting the highest number of imported malaria cases; >80% of cases are caused by *P. falciparum*, the species responsible for almost all severe cases and death in travelers. African travelers are particularly at risk of acquiring malaria when visiting friends and relatives. Together with African residents who declare malaria during a stay in France, African travelers accounted for 57.5% of imported malaria cases in our series. These populations, either immune or possessing residual immunity, are less likely to acquire severe cases of malaria (*9*), which may explain why the total proportion of severe cases in our series, fluctuating according to years from 2.5% to 5.3% (mean 3.8%), appears lower than those reported in previous studies (*3–5,7,10–12*).

We identified 7 risk factors independently associated with severe imported *P. falciparum* malaria; 4 were the same as those associated with fatal imported malaria (older age, being a native from an area where malaria is nonendemic, infection occurring in East Africa, and absence of appropriate chemoprophylaxis), and 3 were new risk factors (first visit was to a general practitioner, time to diagnosis 4–12 days, and diagnosis during the fall–winter season). Increasing age is a well-recognized risk factor for severe (*3–6,17*) and fatal *P. falciparum* malaria (*2,4,14,18,19*). For persons ≥45 years of age, age appeared more as an increased risk factor rather than a gradual increase over the entire age spectrum. This finding is contrary to what has already been reported for death, or for severe cases in another study, which included only

Table 4. Distribution of imported *Plasmodium falciparum* malaria cases by country of acquisition, France, 1996–2003

Country	*P. falciparum* malaria cases	No. (%) severe cases
Comoros	2,017	28 (1.4)
Cameroon	2,707	76 (2.8)
Congo	885	25 (2.8)
Guinea	823	24 (2.9)
Central African Republic	728	25 (3.4)
Côte d'Ivoire	4,623	160 (3.5)
Togo	604	21 (3.5)
Ghana	194	7 (3.6)
Benin	1,012	39 (3.8)
Mali	2,124	83 (3.9)
Gabon	671	32 (4.8)
Senegal	2,234	108 (4.8)
Mauritania	96	5 (5.2)
Burkina Faso	740	41 (5.5)
Madagascar	432	34 (7.8)
Niger	152	12 (7.9)
Tanzania	38	4 (10.5)
Guinea-Bissau	50	6 (12)
Nigeria	123	15 (12.2)
Mozambique	29	4 (13.8)
Kenya	101	16 (15.8)
Equatorial Guinea	31	5 16.1)
Djibouti	12	2 (16.7)
Cape Verde	4	1 (25)
Other	1,458	59 (4.1)
Total	21,888	832 (3.8)

European patients (*2*,*3*). Severe malaria was particularly frequent among nonimmigrants, as previously reported in a smaller series (*7*–*9*,*12*). These results are consistent with the hypothesis of persistent acquired immunity, even after several years of nonexposure (*12*), which may partly protect African travelers from severe malaria (*9*,*12*).

Genetic factors may also partly explain the relative protection of African travelers compared with Europeans, as noted by Lewis et al. (*8*). Five countries, Côte d'Ivoire, Cameroon, Senegal, Mali, and the Comoros Islands, accounted for the largest numbers of malaria cases; case severity rates varied between 1.4% and 4.8%. Travelers returning from the Comoros Islands were almost exclusively migrants who were visiting friends and relatives, which may explain the particularly low proportion of severe cases observed for this country. Conversely, countries in eastern Africa such as Djibouti, Kenya, Mozambique, and Tanzania accounted for fewer cases but displayed a disproportionately high number of severe cases (10.6%–16.7%). This observation confirmed what has been reported for CFR (*2*).

As suggested by a preliminary study (*17*), a relationship between *P. falciparum* drug-resistance level and severity of imported cases may exist. Because countries in eastern Africa usually harbor high proportions of drug-resistant *P. falciparum* strains (*20*,*21*), further specific studies are required to assess this point. The risk of severe malaria was higher when antimalarial chemoprophylaxis was absent or inappropriate. These results are in agreement with previous observations (*5*,*6*,*8*) and advocate strengthening the message of prevention through the use of antimalarial chemoprophylaxis. Even when this prophylaxis fails to prevent malaria because of lack of observance, drug resistance, or pharmacologic hazards, antimalarial

Table 5. Factors independently associated with severe malaria among patients treated for *Plasmodium falciparum* malaria in hospitals, France, 1996–2003*

Variables	Odds ratio (95% confidence interval)	p value
Age group, y		
≤15	1	<0.0001
16–30	0.9 (0.7–1.2)	
31–45	1.06 (0.8–1.3)	
46–60	1.8 (1.4–2.3)	
>60	2.7 (2.0–3.6)	
Origin and residence		
African travelers	1	<0.0001
African residents	1.5 (1.1–1.9)	
European travelers	3.2 (2.6–3.8)	
European expatriates	3.7 (2.9–4.9)	
Others	1.9 (1.5–2.6)	
Region of malaria acquisition		
Western Africa	1	<0.0001
Central Africa	0.8 (0.7–0.9)	
Eastern Africa	2.6 (1.7–4.1)	
Austral Africa	1.1 (0.6–2.2)	
Madagascar and Comoros Islands	0.7 (0.5–0.9)	
Others	0.9 (0.6–1.5)	
Chemoprophylaxis		
Appropriate drugs†	1	0.001
No chemoprophylaxis	1.3 (1.1–1.5)	
Inappropriate drugs‡	1.5 (1.2–1.9)	
Place of first visit		
Hospital	1	
General practitioner	1.4 (1.2–1.7)	<0.0001
Time between onset and diagnosis, d		
≤1	1	<0.0001
2–3	0.9 (0.8–1.2)	
4–6	1.6 (1.3–1.9)	
7–12	1.5 (1.1–1.8)	
>12	0.7 (0.5–0.9)	
Symptom onset		
After return to France	1	
Before return to France	1.2 (1.01–1.5)	0.03
Season of diagnosis		
Spring–summer	1	
Fall–winter	1.3 (1.2–1.5)	<0.0001

*N = 21,888.
†Appropriate chemoprophylactic drugs were mefloquine, atovaquone-proguanil, doxycycline, and chloroquine-proguanil.
‡According to national recommendations, inappropriate chemoprophylactic drugs were chloroquine, proguanil, pyrimethamine, and sulfadoxine-pyrimethamine.

chemoprophylaxis may confer a degree of protection against the severe form of malaria.

Management of patients who had uncomplicated malaria was not standardized among the different hospitals of our network. Depending on local procedures or on individual evaluations, patients were hospitalized, usually for the duration of their treatment, or were treated on an outpatient basis. Nevertheless, each patient with severe malaria needed to be hospitalized in an intensive care unit (exceptions to this rule depend on local medical practice). The odds of severe malaria developing were increased by 40% when the patient's initial visit was to a general practitioner rather than to a hospital. This effect remained after controlling for time to diagnosis and suggests that this association was not due to a simple delay of diagnosis.

Time between onset of symptoms and diagnosis of 4–12 days was associated with an increased risk of severe malaria. Shorter delays of diagnosis enabled prompt treatment of malaria episodes and probably prevented their potential evolution towards severity. Diagnosis >12 days postsymptom onset usually reflected controlled parasitemia and uncomplicated malaria. These data emphasized once again the need for early diagnosis and prompt therapy. The higher severity found during the fall–winter season has been related previously to a potential mismanagement of malaria patients initially misdiagnosed with influenza (*22*,*23*). However, delay in diagnosis does not totally explain higher severity because the association between seasons and severity was not reduced after time to diagnosis was controlled for in multivariate analysis. Moreover, the influenza season in France is shorter than the fall–winter season considered in our analysis. Multivariate analysis in another study (E. Seringe et al., unpub. data) showed that malaria episodes around the end of the transmission season in areas where malaria is endemic were significantly associated with an increased risk of death (OR for each additional month away from the end of the malaria season 0.75, 95% CI 0.64–0.87; p<0.001). If one considers the geographic origin of the largest number of imported malaria cases, the end of the transmission season matched the French winter season. An additional factor, symptom onset before return to France, was marginally independently associated with severe malaria (OR 1.20, 95% CI 1.01–1.50; p = 0.03). Repatriations for medical reasons, including malaria infections, may partially explain this result.

This study has several limitations. Our surveillance network accounted for only 50%–55% of total malaria cases (uncomplicated, severe, or fatal) imported to France. Two annual exhaustive studies (National Quality Control Survey [*24*]) and F. Legros (unpub. data) suggest that our corresponding sites had a correct representation of patients. Thus, it is unlikely that factors associated with severity in imported malaria would be different among cases not seen in our network. From 2000 through 2003, the CFR in patients infected with *P. falciparum* and the percentage of severe cases have increased from 0.3% to 0.7% (p<0.0001) and from 2.5% to 4.5% (p = 0.3) respectively, whereas the CFR of severe cases remained stable (from 14.3% to 15.3%). These figures may be caused by fluctuations over time but may also be partially explained by the revision of WHO criteria for severe malaria in 2000. This revision may have led to a better categorization of severe cases, judging by the increased odds of death associated with severity after the 2000 reclassification (OR 1.84, 95% CI 1.20–2.90). However, no interaction was identified between year of diagnosis and relevant associations and all analyses were adjusted for the year of diagnosis.

Biological data were not introduced in multivariate analysis, first, because of a large number of missing data (59% and 60% of missing data in severe cases for platelet counts and hemoglobinemia, respectively) and, second, because parasitemia (10% of missing data), hemoglobin, and platelet counts are directly or indirectly part of the definition of severe malaria. Thus, parasitemia, although strongly associated with severe forms of malaria in univariate analysis, was not introduced in the final multivariate model because it is in the causal pathway between several factors and disease. For instance, delay to diagnosis leads to high parasitemia, which itself leads to severe forms of malaria. Adjusting for high parasitemia in the final model would make the relationship between delay to diagnosis and severe malaria disappear.

Treatment of severe malaria may have varied according to the physicians. Detailed French guidelines for the management of severe *P. falciparum* malaria were available (*25*). These guidelines strongly recommended a quinine-loading dose but did not recommend exchange transfusion. Intravenous artesunate was not used in France during the study period.

The clinical course of *P. falciparum* malaria is unpredictable and may result in severe illness and death. Although the acquisition of *P. falciparum* malaria among travelers to countries where malaria is endemic can never be completely avoided, our data suggest that severe malaria may largely be prevented. Pretravel health advice promoting the compliant use of antimalaria chemoprophylaxis for every traveler, with a particular focus on nonimmune travelers and elderly persons, is essential. In addition, increased vigilance of travelers in reporting symptoms and of physicians in providing prompt diagnosis and treatment is required to reduce any delay in patient management.

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Plasmodium knowlesi Malaria in Children

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Plasmodium knowlesi can cause severe malaria in adults; however, descriptions of clinical disease in children are lacking. We reviewed case records of children (age <15 years) with a malaria diagnosis at Kudat District Hospital, serving a largely deforested area of Sabah, Malaysia, during January–November 2009. Sixteen children with PCR-confirmed *P. knowlesi* monoinfection were compared with 14 children with *P. falciparum* monoinfection diagnosed by microscopy or PCR. Four children with knowlesi malaria had a hemoglobin level at admission of <10.0 g/dL (lowest minimum level 6.4 g/dL). Minimum level platelet counts were lower in knowlesi than in falciparum malaria (median 76,500/μL vs. 156,000/μL; p = 0.01). Most (81%) children with *P. knowlesi* malaria received chloroquine and primaquine; median parasite clearance time was 2 days (range 1–5 days). *P. knowlesi* is the most common cause of childhood malaria in Kudat. Although infection is generally uncomplicated, anemia is common and thrombocytopenia universal. Transmission dynamics in this region require additional investigation.

The simian malaria parasite *Plasmodium knowlesi* is increasingly recognized as a frequent cause of potentially fatal human malaria in adults in Malaysian Borneo (*1–4*). The infection has also been reported in peninsular Malaysia (*5*) and in other Southeast Asian countries, including Thailand (*6*,*7*), Myanmar (*8*,*9*), Vietnam (*10*), the Philippines (*11*), Indonesian Borneo (*12–14*), and Singapore (*15*,*16*). Until recently, *P. knowlesi* had been almost uniformly misdiagnosed by microscopy as *P. malariae* because of its morphologic similarities, leading to underestimations of prevalence (*1*,*17*). Accurate diagnosis therefore requires molecular methods.

The clinical and laboratory features of *P. knowlesi* infections in adults have been described in Kapit, Sarawak, where 107 (70%) of 152 adults with malaria were infected with *P. knowlesi* (*3*). Although *P. knowlesi* malaria was diagnosed in 8 children, the clinical and laboratory features were not described. All previously reported *P. knowlesi* infections that caused clinical disease have been in adults (*1*,*2*,*6*,*8*,*11–13*,*15*,*18–20*). In malaria caused by *P. falciparum* (*21*) and *P. vivax* (*22*), the 2 species that cause the greatest number of human malaria cases, well-described differences exist between adults and children in terms of the clinical epidemiology, disease spectrum, and laboratory manifestations of disease. We report the demographic, clinical, and laboratory features of *P. knowlesi* infection in children in Kudat, Sabah, a rural coastal farming area with little remaining primary rainforest, an epidemiologic setting that contrasts with the previously described forested areas of Sarawak.

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Methods

Study Setting

The study was conducted at Kudat District Hospital (KDH) on the northeast tip of Sabah, Malaysia, a coastal rural area which has been largely deforested. KDH services 5 subdistricts (Tigapapan, Dualog, Matunggung, Tambuluran, and the island of Banggi), with a total population of 85,000 persons. The Rungus are the most

common ethnic group on the mainland, and minority groups include ethnic Chinese, Bajaus, Dusuns, and Balabaks.

Ministry of Health policy in Sabah requires that all patients with a blood film result that indicates malaria be admitted to the hospital and discharged only after smear results are negative for 2 consecutive days. Since January 2009, in response to increasing reports of *P. knowlesi* infections and the difficulties of diagnosing this species by microscopy, it became policy at KDH to send slides that had been determined by microscopy to show presence of *P. malariae* to the Sabah State Reference Laboratory for PCR confirmation. In addition, KDH sends ≈15% of all blood films positive for other *Plasmodium* spp. for PCR confirmation.

Retrospective Case Review

We retrospectively searched laboratory microscopy records for all blood smear results positive for *Plasmodium* spp. during January 1–November 30, 2009. The patient's age, sex, ethnicity, and address were recorded for all positive samples. Microscopy results were matched with PCR results.

Medical records were retrieved for all children <15 years of age who had received a diagnosis of malaria on the basis of microscopy results. Demographic, clinical, and laboratory details were extracted by using standardized data forms, which also included disease response to antimalarial treatment. Parasite clearance time was defined as the number of days until negative smear. Anemia and severe anemia were defined as hemoglobin levels <11 g/dL and <7.1 g/dL (*3*), respectively.

Laboratory Procedures

Blood films were examined by experienced laboratory microscopists at KDH with the parasite count being classified in most on a scale of 1 to 4 (1 = 4–40 parasites/μL, 2 = 41–400 parasites/μL, 3 = 401–4,000 parasites/μL, 4 = >4,000 parasites/μL), with accurate quantitation per microliter being recorded for most blood films that showed *P. knowlesi,* but for only a limited number that showed *P. falciparum*. Hemoglobin level and leukocyte and thrombocyte counts were measured on site by using automated systems (Sysmex XT1800 [Sysmex Corp., Mundelein, IL, USA] and CELL-DYN Sapphire [Abbott Diagnostics, Abbott Park, IL, USA]) At the Sabah State Reference Laboratory, parasite DNA was extracted, and nested PCR was performed for *P. falciparum, P. vivax, P. malariae, P. ovale*, and *P. knowlesi* by methods described (*1*,*23*).

Statistical Analysis

Data were analyzed by using Stata statistical software, version 10 (StataCorp LP, College Station, TX, USA). Proportions were compared by using the χ^2 or Fisher exact test. Normally distributed and non–normally distributed variables were compared by using Student *t* test and Wilcoxon rank-sum test, respectively. For comparison between *P. falciparum* and *P. knowlesi* cases, the analysis included children with PCR-confirmed *P. knowlesi* infection, and children with *P. falciparum* infection diagnosed by either microscopy or PCR. Children with mixed *Plasmodium* infections were excluded from analysis, as were children whose medical records could not be located.

Results

Malaria in All Age Groups

From January 1 through November 30, 2009, 220 patients at KDH were given a diagnosis of malaria on the basis of microscopy results (Figure 1). Of these, 196 (89%) had *P. malariae* monoinfection or mixed infection. PCR was performed on samples from 157 (80%) of these patients and results were positive for *P. knowlesi* in 137 (87%); 125 (91%) of these were *P. knowlesi* monoinfections. For the remaining 20 patients who had been given a diagnosis of *P. malariae* monoinfection or mixed infection by microscopy, PCR found undifferentiated *Plasmodium* spp., negative results for *Plasmodium* spp., and 7 cases of *P. falciparum* infection.

To estimate the final numbers of malaria species, we used positive PCR results when possible and microscopy results when PCR had not been performed (replacing *P. malariae* with *P. knowlesi*). Microscopy results were also used if PCR result was negative (5 *P. knowlesi* infections and 1 *P. knowlesi/P. falciparum* infection) or if PCR result was positive for *Plasmodium* spp. (7 *P. knowlesi* infections). Using this method, we found 172 (78%) *P. knowlesi* monoinfections, 29 (13%) *P. falciparum* monoinfections, and 19 (9%) mixed infections (Figure 1). A greater proportion of patients with *P. knowlesi* malaria were male (123/172 [72%]) than were those with *P. falciparum* malaria (15/29 [52%]; p = 0.03). Median age was higher for those with *P. knowlesi* infection (median 32 years, interquartile range [IQR] 19–49 years) than for those with *P. falciparum* infection (median 11 years, IQR 6–30 years).

Malaria in Children

Of 220 patients with positive results by microscopy, 41 (19%) were <15 years of age. Microscopy showed that 24 (59%) children had *P. malariae* monoinfection, 10 (24%) had *P. falciparum* monoinfection, and 7 (17%) had mixed infections (Figure 2). Samples from 17 children with *P. malariae* monoinfection underwent PCR; 16 (94%) showed *P. knowlesi,* and 1 showed mixed *P. knowlesi/P. vivax* infection. Again, final numbers of malaria species were

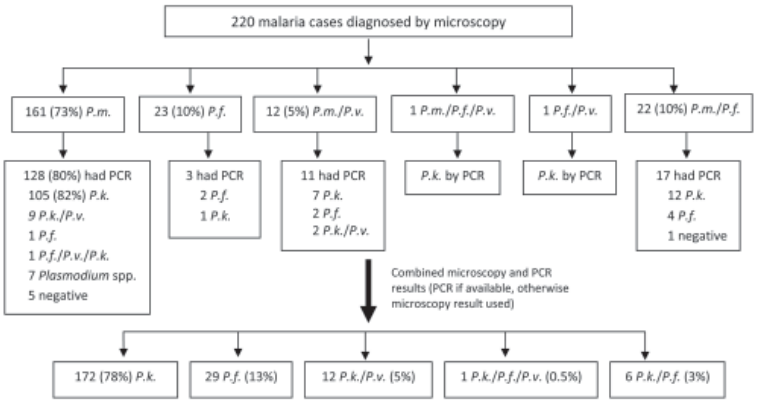

Figure 1. Distribution of malaria cases diagnosed by microscopy and PCR among all age groups, Kudat, Malaysia, January 1–November 30, 2009. *P.m.*, *Plasmodium malariae*; *P.f., P. falciparum*; *P.v., P. vivax*; *P.k.*, *P. knowlesi*; neg, negative.

estimated by using PCR results (if available) or microscopy (if PCR had not been performed). Accordingly, 24 cases (59%) were *P. knowlesi*, 15 (37%) were *P. falciparum,* and 2 were mixed *P. knowlesi/P. vivax* infections. Children thus represented 14% (24/172) of all cases of *P. knowlesi* monoinfection.

Further analysis was confined to those children with PCR-confirmed *P. knowlesi* monoinfection and children with *P. falciparum* monoinfection diagnosed by either microscopy or PCR. Medical records were unavailable for 2 children (1 with *P. knowlesi* and 1 with *P. falciparum* infection), leaving 16 children with PCR-confirmed *P. knowlesi* infection and 14 with *P. falciparum* infection for comparison (Table 1).

Demographic Characteristics of Children with *P. knowlesi* Malaria

Half of the children with PCR-confirmed *P. knowlesi* malaria were male (Table 1). The mean age was 8.9 (95% confidence interval 7.6–10.3) years. The youngest child with *P. knowlesi* infection was 4 years; all others (15/16; 94%) were 7–14 years of age. In contrast, children with *P. falciparum* malaria were significantly younger, with a mean age of 5.4 years (95% confidence interval 3.5–7.3; range 9 months–11 years; p = 0.002).

Clinical Features of *P. knowlesi* and *P. falciparum* Malaria

All children had fever or a history of fever. The duration of fever before hospital admission appeared shorter with knowlesi malaria than with falciparum malaria (median 4 days vs. 6.5 days), although this difference was not statistically significant. Although documentation of clinical features was limited, no child was in a state of shock, and no child was reported to be dyspneic, to have bleeding complications, or to have any other clinical feature or laboratory results that indicated severe malaria (*24*). None of the children who had either knowlesi or falciparum malaria died.

Anemia

In 11 (69%) children with *P. knowlesi* malaria, the parasite density was accurately determined at hospital admission, with a median of 2,240 (IQR 480–7,200) parasites/μL. Only 2 children with *P. falciparum* malaria had parasite densities accurately determined at admission (200 and 1,600 parasites/μL). Anemia was common with *P. knowlesi* and *P. falciparum* infections. At admission, 9 (56%) children with *P. knowlesi* infection were anemic (hemoglobin level <11.0 g/dL), and 4 (25%) had a hemoglobin level <10 g/dL. All children with *P. knowlesi* infection had a hemoglobin minimum level <11.0 g/dL, and 10 (63%) had a hemoglobin minimum level <10 g/dL. The lowest hemoglobin level was found in an 8-year-old boy: 8.3 g/dL at admission, 6.4 g/dL on day 3, and 7.2 g/dL on day 6, the day before discharge. His parasite count at

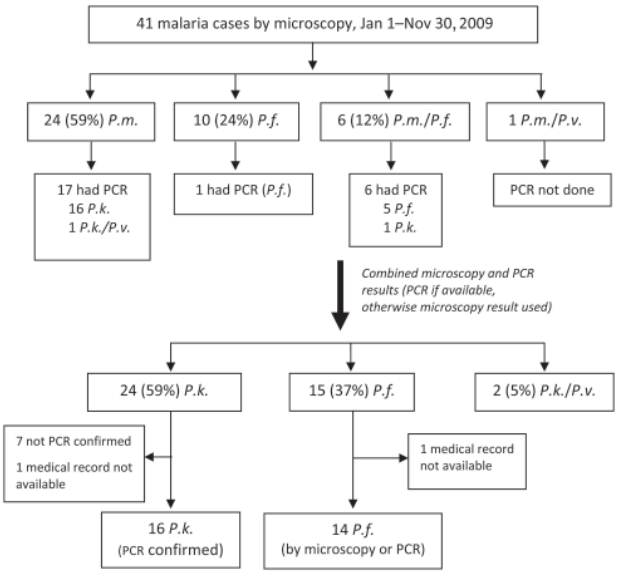

Figure 2. Distribution of malaria cases diagnosed by microscopy and PCR among children <15 years of age, Kudat, Malaysia, January 1–November 30, 2009. *P.m.*, *Plasmodium malariae*; *P.f., P. falciparum*; *P.v., P. vivax*; *P.k., P. knowlesi*.

Table 1. Demographic data, clinical features of infection, and laboratory values in 14 cases of *Plasmodium falciparum* malaria and 16 cases of PCR-confirmed *P. knowlesi* malaria in children, Kudat, Malaysia, January 1–November 30, 2009*

Characteristic	*P. knowlesi*, n =16	*P. falciparum*, n =14	p value
Age, y			
Mean (SD)	8.9 (2.5)	5.4 (3.2)	**0.002**
Range	4–14	0.75–11	
Male sex, no. (%)	8 (50)	7 (50)	1.00
Living in Banggi, no. (%)	0 (0)	10 (71.4)	**<0.001**
Duration of fever, d, median (IQR)	4 (3–6)	6.5 (4–7)	0.12
Examination findings at admission			
Temperature, °C, median (IQR)	37.9 (37.0–39.1)	37.0 (37.0–37.4)	0.07
Heart rate, beats/min, mean (SD)	110 (24)	119 (15)	0.24
Respiratory rate, beats/min, mean (SD)	27.8 (3.9)	32 (3.8)	**0.01**
Systolic blood pressure, mm Hg, mean (SD)	105 (8.3)	98 (11.2)	0.06
Laboratory results			
Hemoglobin d 0, g/dL, median (IQR)	10.7 (10.0–11.4)	8.5 (6.1–10.5)	**0.001**
Hemoglobin minimum level, g/dL, median (IQR)	9.7 (8.4–10.2)	7.15 (6.1–9.3)	**0.035**
Day of hemoglobin minimum level, mean (SD)	2.6 (1.03)	1.5 (1.3)	**0.022**
Lymphocyte count d 0, $\times 10^3$/μL, median (IQR)	2.0 (1.7–2.3)	2.5 (2.0–4.6)	0.09
Lymphocyte minimum level, $\times 10^3$/μL, median (IQR)	1.6 (1.3–2.2)	2.4 (1.9–3.9)	**0.01**
Day of lymphocyte minimum level, median (IQR)	1 (0–1)	1.5 (0–3)	0.19
Thrombocyte count d 0, $\times 10^3$/μL, median (IQR)	89.5 (72.0–118.5)	171.5 (98–271)	**0.038**
Thrombocyte count minimum level, $\times 10^3$/μL, median (IQR)	76.5 (68.5–110.0)	156 (98–227)	**0.014**
Day of thrombocyte count minimum level, median (IQR)	1 (1–1)	0.5 (0–2)	0.67

*IQR, interquartile range. **Boldface** indicates statistical significance.

admission (14,400 parasites/μL) was the highest recorded in this study.

In addition, severe anemia (hemoglobin 6.0 g/dL at admission; minimum level 4.9 g/dL on day 1 requiring transfusion) occurred in a second child with positive results for *P. knowlesi* by microscopy (this child was not included in the final analysis because of lack of PCR confirmation). Among children with *P. knowlesi* infection, a lower hemoglobin minimum level was associated with higher parasite density at admission (p = 0.001). All children with *P. falciparum* infection had hemoglobin levels at admission <11.0 g/dL; 10 (71%) had hemoglobin levels <10 g/dL, and 5 (36%) had severe anemia (hemoglobin <7.1 g/dL). Severe anemia developed in a sixth child after admission. Four children received transfusions, with hemoglobin levels of 4.8–6.1 g/dL at admission. Children with *P. knowlesi* malaria took longer to reach their hemoglobin minimum level than did children with *P. falciparum* malaria (2.6 vs 1.5 days; p = 0.02).

Thrombocytopenia

All children with *P. knowlesi* malaria had thrombocytopenia. Fifteen children (94%) had a platelet count <150,000/μL at admission, and the remaining child exhibited thrombocytopenia within 1 day. The lowest platelet count recorded was 28,000/μL. Platelet count was not correlated with hemoglobin level. Thrombocytopenia was less common in children with *P. falciparum*; 7 (50%) had platelet counts <150,000/μL at admission or during hospitalization and the lowest platelet count was 47,000/μL. Children with *P. knowlesi* malaria had a lower lymphocyte count minimum than those with *P. falciparum* malaria (1.6 vs. 2.4 $\times 10^3$/μL; p = 0.01).

Response to Treatment

Most (81%) children with *P. knowlesi* malaria were given oral chloroquine and primaquine for 3 days, and these children had a median parasite clearance time of 2 days (Table 2). The longest parasite clearance time with chloroquine and primaquine was 5 days in the aforementioned 8-year-old boy with the highest admission parasitemia level (14,400/μL) and the lowest minimum hemoglobin level (6.4 g/dL). Three children with *P. knowlesi* infection were given oral quinine, and parasites cleared within 2 days. Among the 11 children with *P. knowlesi* and parasite densities accurately assessed at admission, the correlation between parasite density and parasite clearance time was significant (p = 0.002).

Children with *P. falciparum* malaria were given a variety of treatment regimens. Most (71%) received a regimen that contained quinine, 7 (50%) children received sulfadoxine/pyrimethamine, 6 (43%) received primaquine, and 5 (36%) were given chloroquine. Only 5 (36%) received artemisinin-based combination therapy. Parasite clearance times were significantly slower among children with *P. falciparum* malaria, with a median of 5 days until the first negative smear; in 4 children (29%), it took ≥10 days for parasites be cleared. Children with *P. knowlesi* malaria had significantly shorter hospital admissions (median 4 days, IQR 4–5 days) than did

Table 2. Malaria treatment according to *Plasmodium* species and response to treatment in children, Kudat, Malaysia, January 1–November 30, 2009*

Species and treatment regimen	No. (%) patients	Days until negative smear†
P. knowlesi, n = 16		
3 days CQ/PQ	13 (81)	2 (2–3, 1–5)
Oral Q	2 (13)	2, 2
IV Q	1 (6)	2
All		2 (2–3, 1–5)
P. falciparum, n = 14		
3 days CQ/PQ → oral Q	2 (14)	5, 5
Oral Q → AL	1 (7)	3
SP/PQ → AM	1 (7)	8
SP/PQ	2 (14)	1, 2
SP/PQ → CQ/Q → AL	1 (7)	3
SP/PQ → CQ/IV Q → AL	1 (7)	5
SP/PQ → CQ/IV Q → oral Q	1 (7)	3
SP/PQ → oral Q → IV Q → oral Q	1 (7)	16
IV Q → oral Q	2 (14)	1, 10
IV Q → oral Q/PQ	1 (7)	11
Oral Q/PQ → AM	1 (7)	10
All		5 (3–10, 1–16)‡

*IQR, interquartile range; CQ, chloroquine; PQ, primaquine; Q, quinine; IV, intravenous; AL, artemether/lumefantrine; SP, sulfamethoxazole/pyrimethamine; AM, artesunate/mefloquine.
†Values are mean (IQR, range) by category or days by patient.
‡p value = 0.009 for days until negative smear for *P. falciparum* vs. *P. knowlesi*.

children with *P. falciparum* malaria (median 7 days, IQR 5–10 days).

Discussion

P. knowlesi was the most common cause of malaria in adults and children in the Kudat region in Sabah, Malaysian Borneo. Although those with *P. knowlesi* infections had an older age distribution than did those with *P. falciparum* infections, the species still caused 63% of all malaria cases among children <15 years. Although nearly all previous reported cases of knowlesi malaria have been in adults, 14% of all knowlesi cases in Kudat occurred in children. In children, *P. knowlesi* most often caused uncomplicated malaria, which responded well to chloroquine. Nevertheless, knowlesi malaria was associated with substantial illness in children, with all PCR-confirmed *P. knowlesi*–infected children being anemic at admission or during hospital stay.

In the only other report of knowlesi malaria in children, a prospective study of adult knowlesi malaria in Sarawak reported the exclusion of 8 children <15 years with *P. knowlesi* infection, comprising 7% of all knowlesi cases (*3*). The clinical and laboratory features of *P. knowlesi* malaria in children have not been described. Consistent with the reported features of *P. knowlesi* malaria in adults (*3*), the disease in most children was uncomplicated. In adults with knowlesi malaria, increasing age is a risk factor for severe disease. Although the numbers are small, none of the children had severe manifestations of knowlesi malaria that have been reported in adults (*3*), such as acute lung injury or acute renal failure. In falciparum malaria, these conditions are also largely confined to adults (*21*); anemia, coma, and acidosis-related respiratory distress are the major manifestations of severe falciparum malaria in children. No child with knowlesi malaria exhibited coma or respiratory distress; however, anemia developed in all children with knowlesi malaria, with the hemoglobin concentration in 1 patient (6%) falling to <7.0 g/dL. Anemia was more common in children than has been previously described in knowlesi malaria in adults (*3*).

Thrombocytopenia was found at admission in nearly all (94%) children with *P. knowlesi* malaria, in contrast to only half of the children with *P. falciparum* malaria. Although the cause is unclear, thrombocytopenia is also nearly universal in infected adults (*3*), which makes it a characteristic feature of *P. knowlesi* infection across all age groups. The role of thrombocytopenia and platelet activation in the pathogenesis of knowlesi malaria requires further investigation.

Most children with *P. knowlesi* malaria had an adequate response to a 3-day regimen of treatment with chloroquine and primaquine, although the mean parasite clearance time of 2 days was longer than the 90% parasite clearance time of 10.3 hours that was recently reported in adults (*25*). One child who received chloroquine and primaquine, and had a high parasite count at admission, required 5 days to clear parasites. Standard Ministry of Health pediatric dosing regimens of chloroquine are used in Kudat; however, posttreatment vomiting or inadequate blood concentrations could not be excluded. Prospective studies that evaluate the response to chloroquine and artemisinin-based combination

therapy in pediatric knowlesi malaria are required. Children with *P. falciparum* malaria received many different treatment regimens and took significantly longer to clear their parasites. Less than half received the recommended artemisinin-based combination therapy, and only after alternative treatments failed. Children with *P. falciparum* malaria had significantly longer hospital stays, likely related at least in part to suboptimal treatment regimens. This finding highlights the importance of increasing the usage of artemisinin-based combination therapy for falciparum malaria in district hospitals in Sabah and elsewhere.

Our study had several limitations. First, PCR was only performed for 73% of cases across all age groups, and this limited our ability to accurately determine the true proportion of disease caused by *P. knowlesi*. Furthermore, although PCR was performed on samples from most children with *P. knowlesi* malaria, only 1 child with *P. falciparum* malaria had PCR performed. Our analysis, therefore, compared children with PCR-confirmed knowlesi malaria to children with falciparum malaria diagnosed by either microscopy or PCR. Some of those children with a diagnosis of falciparum malaria may have actually had knowlesi malaria, and the differences found between these 2 species may therefore be minimum estimates. The retrospective design of our study also limited our ability to collect standardized clinical information.

This study demonstrates that *P. knowlesi* has become the predominant malaria species in the Kudat region, estimated by results of microscopy, PCR, or both, as contributing to 87% of all malaria cases, a higher proportion than that reported elsewhere in Malaysian Borneo. The emerging dominance of *P. knowlesi* in Malaysian Borneo has been hypothesized to result from the following factors: changing patterns of human exposure to monkeys and vectors (*26*) because of deforestation, and potentially reduced competition or cross-species protection from *P. vivax* and *P. falciparum* as a result of a 40-fold reduction in the prevalence of these species in Sabah and Sarawak during 1960–2006 following intensive malaria control efforts (*27*). The paucity of *P. vivax* in Kudat was particularly notable.

Previous reports of adult disease have been from communities adjacent to rainforests (*3*). Kudat is a coastal rural farming area with varied land use and vegetation patterns and with minimal remaining regrowth forest. Although our retrospective study did not gather detailed travel or exposure histories, it is likely that most pediatric infections were locally acquired and that infections with *P. knowlesi* did not occur solely in those spending time in forested areas. Macaque monkeys, the natural hosts of *P. knowlesi*, are widely distributed in different habitats throughout the Kudat area and are frequently domesticated. The major vectors of *P. knowlesi* in forested areas of Sarawak, *Anopheles latens* mosquitoes, disappear with deforestation, but vectors capable of transmitting *P. knowlesi, An. balabacencis* mosquitoes (*26*), do persist at lower densities in largely deforested areas of Sabah (*28*,*29*). In notable contrast to *P. falciparum* malaria, pediatric knowlesi malaria was restricted to children of school age. Further studies will be required to characterize the transmission patterns, vectors, and risk factors for *P. knowlesi* in deforested areas of Malaysia.

Conclusions

P. knowlesi is the most common cause of malaria in adults and children in the Kudat region of Sabah, a rural coastal deforested region. Consistent with previous studies in adults (*3*), we found that *P. knowlesi* in children most often caused uncomplicated malaria that responded adequately to chloroquine and primaquine. Anemia was common in children and knowlesi infection was associated with moderately severe anemia. Thrombocytopenia was universal and is characteristic of knowlesi malaria across all age groups. Larger prospective clinical studies are needed to describe more fully the epidemiology of *P. knowlesi* malaria in children, the full spectrum of clinical disease and the transmission patterns in nonforested areas such as Kudat.

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Travel-related Dengue Virus Infection, the Netherlands, 2006–2007

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To assess the incidence of and risk factors for clinical and subclinical dengue virus (DENV) infection, we prospectively studied 1,207 adult short-term travelers from the Netherlands to dengue-endemic areas. Participants donated blood samples for serologic testing before and after travel. Blood samples were tested for antibodies against DENV. Seroconversion occurred in 14 (1.2%) travelers at risk. The incidence rate was 14.6 per 1,000 person-months. The incidence rate was significantly higher for travel during the rainy months. Dengue-like illness occurred in 5 of the 14 travelers who seroconverted. Seroconversion was significantly related to fever, retro-orbital pain, myalgia, arthralgia, and skin rash. The risk for DENV infection for short-term travelers to dengue-endemic areas is substantial. The incidence rate for this study is comparable with that in 2 other serology-based prospective studies conducted in the 1990s.

Dengue virus (DENV) is a flavivirus transmitted through the bite of an infected *Aedes* spp. mosquito. The disease is endemic to tropical and subtropical regions and each year affects ≈100 million persons worldwide. Most cases are reported from Southeast Asia and Central and South America (*1*,*2*).

The incubation period for DENV infection is 3–14 days. Manifestations are nonspecific and range from asymptomatic to severe (*3*,*4*). Most infections are benign, with few deaths. In 2%–3% of patients, dengue hemorrhagic fever develops (*5*).

DENV has 4 distinct serotypes. Primary infection with 1 serotype confers lifelong immunity to that serotype but increases the risk for severe dengue illness after secondary infection with another serotype (*6*). No specific therapy exists for dengue.

During the past few decades, dengue has emerged in tropical and subtropical countries worldwide (*1*,*7*). In addition, the number of reported symptomatic DENV infections among international travelers has increased (*5*,*8*,*9*). This increase may reflect increased incidence of dengue among travelers, increased number of travelers to areas in which dengue is endemic, increased awareness of the disease among physicians, or a combination of these factors.

However, valid research on travelers' risk for DENV infection is scarce. To our knowledge, only 2 prospective studies, performed in the 1990s (*10*,*11*), have been conducted. Most other epidemiologic studies are retrospective and focus on symptomatic patients who seek care at a clinical site, thus disregarding the nonspecific nature of the infection and the increased number of travelers to dengue-endemic areas (*5*,*8*,*9*,*12*,*13*).

We prospectively estimated the prevalence and incidence of DENV infection and its risk factors. Our findings are based on serologic testing in a cohort of short-term travelers from the Netherlands to dengue-endemic areas.

Methods

Study Population

We prospectively studied persons attending the travel clinic of the Public Health Service Amsterdam during

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October 1, 2006–September 30, 2007. All persons ≥18 years of age were eligible if they planned to travel for 1–13 weeks to ≥1 countries to which dengue is endemic. Dengue endemicity was based on information from The Global Infectious Diseases and Epidemiology Online Network (*14*).

All participants were seen by a medical doctor or nurse who specialized in travel medicine. They received vaccinations, a prescription for antimalarial chemoprophylaxis if required, and oral and written information about how to avoid acquiring travel-related diseases, including mosquito-borne infections, according to Dutch National Guidelines on Traveler's Health Advice (*15*).

Survey Methods

Before departure (just before they received the required vaccinations) and 2–6 weeks after return, participants donated venous blood samples for serologic testing. Before departure,they completed a standard questionnaire to collect data on sociodemographic factors, including travel history, vaccination status, and the purpose of travel (tourism, work/education, or visits to friends and/or relatives).

Participants were given a digital thermometer and asked to take their temperature (axillary or rectally) when feeling feverish. They kept a structured travel and posttravel diary to record symptoms and signs of dengue illness (fever, myalgia, arthralgia, headache, retro-orbital pain, skin rash, signs and symptoms of gastrointestinal disease), use of insect repellent containing N,N-diethyl-meta-toluamide (DEET), and use of mosquito netting. Participants were asked to report both the presence and absence of symptoms and preventive measures on a daily basis; after travel, the diary was checked by a registered nurse in the participant's presence.

To encompass the incubation period of DENV infection, we used data from the third day after arrival at the travel destination until 2 weeks after return from travel. With respect to symptomatic outcome, we used the term travel-related to refer to this period.

Destinations were grouped into regions by using the classification of the United Nations Development Agency (*16*). Travel duration was recorded as the total days spent in areas meeting the inclusion criteria.

The Medical Ethics Committee of the Academic Medical Centre Amsterdam approved the study protocol. Participants were included only if they provided informed and written consent.

Laboratory Methods

Blood samples were immediately stored at 6°C, then centrifuged and frozen at –80°C within 24 h after collection. After all study participants had returned, all posttravel serum samples were thawed and tested for immunoglobulin (Ig) M antibodies to DENV antigen by using an IgM-capture ELISA and for IgG antibodies to DENV antigen serotypes 1, 2, 3, and 4 by using an indirect ELISA (Panbio Diagnostics, Brisbane, Queensland,Australia) according to the manufacturer's instructions. For participants whose posttravel sample yielded positive test results for anti-DENV IgM or IgG, pretravel samples were also tested for anti-DENV IgM or IgG, respectively.

The ELISA for anti-DENV IgM has a sensitivity of 87%–100% and a specificity of 96% (*17–19*). The ELISA for anti-DENV IgG has a sensitivity of 99%–100% and a specificity of 96%–98% (*17*,*20*). These test characteristics concern the use of paired serum samples.

Serology suggestive for previous DENV infection was defined as an anti-DENV IgG >11 Panbio units in both the pretravel and the posttravel sample. The posttravel-to-pretravel anti-DENV IgG ratio was <4:1.

We defined seroconversion indicating recent DENV infection. Criteria were as follows: 1) posttravel anti-DENV IgM >11 Panbio units with pretravel anti-DENV IgM <11 Panbio units and anti-DENV IgG <11 Panbio units; and/or 2) posttravel anti-DENV IgG >11 Panbio units and pretravel anti-DENV IgG <11 Panbio units, with a posttravel-to-pretravel ratio of ≥4:1; and/or 3) anti-DENV IgG >11 Panbio units in both the pretravel and posttravel sample, with a posttravel-to-pretravel ratio of ≥4:1 (*21*).

Data Analysis

To calculate risk factors for previous DENV infection, we used SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA) to obtain prevalence rates, univariate and multivariate prevalence rate ratios, and 95% confidence intervals (CIs) by using logistic regression modeling. All variables with an overall p value <0.05 in univariate analysis were included in multivariate analysis. Statistical interactions between variables were checked for all variables. If significant ($p<0.05$), they were included in multivariate analysis.

We calculated attack rates of recent DENV infection per 100 travelers by dividing the number of seroconversions by the total number of participants. Incidence rates (IR) per 1,000 person-months were calculated by dividing the number of seroconversions by the total number of months in which participants were at risk for DENV infection. To calculate risk factors for recent DENV infection, incidence rate ratios (IRR) were obtained as conditional maximum-likelihood estimates of the rate ratio, with mid-P exact test, 95% CIs, and p values. We quantified the application of a protective measure against mosquito bites by dividing the number of days the measure was applied by the number of travel days; it was dichotomized by using the mean of this proportion in the total study population as the cutoff.

Travel during the rainy months was assessed by using the midtravel dates. The rainy months per destination were defined as months with an average total precipitation of >100 mm, based on World Weather Information Service data from the United Nations' World Meteorological Organisation (*22*).

We used χ^2 tests to evaluate the predictive value of travel-related signs and symptoms for recent DENV infection. Fever was defined as a body temperature ≥38°C. Dengue-like illness was defined as any episode of fever accompanied by ≥1 of the following: myalgia, arthralgia, headache, retro-orbital pain, or skin rash. A p value <0.05 was considered significant.

Results

Study Population

The study began with 1,276 participants who intended to travel to dengue-endemic areas. Of these, 69 (5.4%) were excluded: 33 were lost to follow-up; 23 had their travel arrangements cancelled; 7 did not supply the posttravel blood donation; 3 changed travel plans such that inclusion criteria were not met; 2 did not complete the structured diary; and for 1, the posttravel sample was lost.

Most of the remaining 1,207 participants were persons of Dutch origin who were traveling for tourism and previously had traveled to dengue-endemic areas (Tables 1, 2). The median travel duration was 21 days, with an interquartile range (IQR) of 16–28. The median interval between return from travel and posttravel blood donation was 25 days (IQR 21–29).

Twenty-six percent of travelers had received yellow fever vaccination for a previous journey, and 23% received a yellow fever vaccination for the current trip, just after blood donation (not shown in Table 1). Only 4 (0.3%) participants had ever received Japanese encephalitis vaccination, all for a previous journey. No one had ever received vaccination against tick-borne encephalitis.

Serologic Results Suggestive for Previous DENV Infection

Serologic results suggestive of previous DENV infection were found for 78 (6.5%; 95% CI 5.2%–8.0%) participants (Table 1). Previous DENV infection was positively correlated with increasing age. The rate was significantly higher for travelers born in a dengue-endemic country, for those who traveled frequently to dengue-endemic countries (all 78 reported ≥1 previous trips to such

Table 1. Characteristics of 1,207 persons who traveled from the Netherlands to dengue-endemic countries and who attended a travel clinic for pretravel health advice, October 1, 2006–September 30, 2007*

		Previous DENV		Univariate analysis		Multivariate analysis	
Characteristic	Total, no. (%)	No.	PR, %	PRR (95% CI)	p value	PRR (95% CI)	p value
No. participants	1,207 (100)	78	6.5	NA			
Sex							
M	521 (43)	29	5.6	1			
F	686 (57)	49	7.1	1.3 (0.81–2.1)	0.27		
Median age, y, IQR	38, 29–51	49, 35–59		1.04 (1.02–1.06)	<0.0001	1.03 (1.01–1.05)	0.003
Country of birth							
Non–dengue-endemic country	1,118 (93)	40	3.6	1		1	
Dengue-endemic country	89 (7)	38	43	20.1 (11.9–34.0)	<0.0001	14.4 (8.1–25.6)	<0.0001
No. times previously traveled to a dengue-endemic country					<0.0001		0.004
0–1	447 (37)	5	1.1	1		1	
2–5	477 (40)	35	7.3	7.0 (2.7–18.0)	<0.0001	5.0 (1.8–13.9)	0.002
≥6	283 (23)	38	13.4	13.7 (5.3–35.3)	<0.0001	6.4 (2.1–19.2)	0.001
Previous travel destinations							
Not Asia	537	31	5.8	1			
Asia	670 (56)	47	7.0	1.2 (0.77–2.0)	0.38		
Not Latin America	661	22	3.3	1		1	
Latin America	546 (45)	56	10.3	3.3 (2.0–5.5)	<0.0001	1.6 (0.85–2.9)	0.15
Not Africa	679	41	6.0	1			
Africa	528 (48)	37	7.0	1.2 (0.74–1.9)	0.50		
Primary purpose of current travel					<0.0001		0.001
Tourism	1,032 (86)	52	5.0	1		1	
Work or education	99 (8)	8	8.1	1.7 (0.76–3.6)	0.20	1.3 (0.52–3.3)	0.57
Visit with friends and/or relatives	76 (6)	18	23.7	5.8 (3.2–10.6)	<0.0001	4.1 (2.0–8.5)	<0.0001
Vaccination status							
Not vaccinated for previous travel	888 (74)	46	5.2	1		1	
Vaccinated for previous travel	319 (26)	32	10.0	2.0 (1.3–3.3)	0.003	1.0 (0.56–1.78)	0.99

*DENV, dengue virus; PR, prevalence rate; PRR, prevalence rate ratio; CI, confidence interval; NA, not applicable; IQR, interquartile range.

Table 2. Characteristics of travelers with serologic evidence of recent dengue virus infection from a cohort of 1,207 persons who traveled from the Netherlands to developing countries, October 1, 2006–September 30, 2007*

Traveler no.: age, y/sex	Country of birth	Destination country (region)	Previous times traveled to developing country	Travel-related signs and symptoms	Dengue-like illness	Vaccinated against yellow fever	IgM-ratio†	IgG-ratio†
1: 33/M	The Netherlands	Philippines (Asia)	2–5	Fever, myalgia, headache, retro-orbital pain, diarrhea	Yes	Never	NA	17.65
2: 62/M	Germany	Indonesia (Asia)	0	Skin rash, diarrhea	No	Never	51.79	38.9
3: 64/M	The Netherlands	Sri Lanka (Asia)	>10	Fever, myalgia, arthralgia, headache, retro-orbital pain, diarrhea	Yes	In 1992	NA	28.3
4: 41/M	The Netherlands	Cambodia, Thailand, Malaysia, Philippines (Asia)	>10	None	No	Never	NA	46.27
5: 36/F	The Netherlands	Thailand, Singapore (Asia)	6–10	Diarrhea	No	Never	3.34	NA
6: 37/F	The Netherlands	Indonesia (Asia)	0	Fever, myalgia, headache, retro-orbital pain, skin rash	Yes	Never	48.66	142.25
7: 28/M	The Netherlands	Vietnam (Asia)	2–5	None	No	Never	2.78	NA
8: 27/M	The Netherlands	India, Nepal (Asia)	2–5	Diarrhea	No	Never	5.11	NA
9: 68/M	The Netherlands	Surinam (Latin America)	1	None	No	In 2000	NA	8.91
10: 26/F	The Netherlands	Argentina, Bolivia, Brazil, Chili, Uruguay (Latin America)	0	Fever, myalgia, arthralgia, headache, skin rash, diarrhea	Yes	For this trip	NA	26.67
11: 23/F	The Netherlands	Surinam (Latin America)	1	Skin rash, diarrhea	No	For this trip	NA	72.82
12: 54/F	The Netherlands	Gambia (Africa)	2–5	Fever, myalgia, arthralgia, headache, retro-orbital pain, diarrhea	Yes	For this trip	NA	26.87
13: 27/M	The Netherlands	Mali, Burkina Faso (Africa)	2–5	Skin rash, diarrhea	No	In 2000	18.15	NA
14: 52/M	The Netherlands	Madagascar (Africa)	6–10	Diarrhea	No	In 1992	8.82	NA

*Ig, immunoglobulin; NA, ratio not available.
†Posttravel to pretravel. If the posttravel sample test results were negative for a type of anti-DENV, the pretravel serum sample was not tested for that type of antibody.

a country), and for those whose purpose was to visit friends or relatives. In multivariate analysis, previous DENV infection was not related to yellow fever vaccination for previous travel because it was confounded by previous trips to a developing country. We found no interactions indicating effect modification.

Serology Suggestive of Recent Dengue Infection

Fourteen participants (1.2%; 95% CI 0.66%–1.9%) had recent DENV infection, of whom 5 seroconverted only for IgM; 7 seroconverted only for IgG, with a posttravel-to-pretravel ratio of ≥4:1; and 2 seroconverted for both IgM and IgG (Table 2). Thus, the attack rate for recent DENV infection was 1.2% (95% CI 0.66%–1.9%). The incidence rate was 14.6 per 1,000 person-months (95% CI 8.3–23.9) (Table 3).

All 14 persons with recent infection were born in non–dengue-endemic countries; 9 were male, and 11 had traveled previously to a dengue-endemic country (Table 3). The median age was 36 years (IQR 27–56 years). The primary purpose of travel was tourism (12 persons) or work/education (2 persons). The median travel duration was 24 days (IQR 19–30).

The incidence rate was significantly higher for travel during the rainy months (23.6 vs. 7.5/1,000 person-months; IRR 3.2, 95% CI 1.01–11.6; p = 0.048). Travel during the rainy months was not related to use of insect repellent containing DEET or use of a mosquito net (odds ratio 1.0; p>0.05).

The incidence rate appeared to be higher for male travelers (22.3 vs. 9.0; IRR 2.5, 95% CI 0.83–8.2; p = 0.11), persons who traveled for work or education (23.6 vs. 13.7; IRR 1.7, 95% CI 0.26–6.8; p = 0.47), and those who used insect repellent containing DEET <45% of travel days (18.6 vs. 9.5; IRR 2.0, 95% CI 0.63–7.2; p = 0.26), but differences were not significant. With regard to age,

Table 3. Attack rates and incidence rates of seroconversions in antibody levels for dengue virus in 1,207 persons who traveled from the Netherlands to dengue-endemic countries, October 1, 2006–September 30, 2007*

Region	No. (%) persons at risk	No. seroconversions	Person-months of travel	Attack rate, % (95% CI)	Incidence rate, %† (95% CI)
All	1,207 (100)	14	959.2	1.2 (0.66–1.9)	14.6 (8.3–23.9)
Asia	569 (47)	8	474.3	1.4 (0.66–2.7)	16.9 (7.8–32.0)
Southeast Asia	375 (31)	6	324.0	1.6 (0.82–3.7)	18.5 (9.4–42.7)
Southern Asia	147 (12)	2	116.4	1.4 (0.034–3.3)	17.2 (0.43–42.4)
East Asia	98 (8)	0	86.2	0	0
Latin America	340 (28)	3	261.3	0.9 (0.23–2.4)	11.5 (2.9–31.3)
South America	219 (18)	3	184.5	1.4 (0.35–3.7)	16.3 (4.1–44.3)
Central America and Caribbean	135 (11)	0	96.0	0	0
Africa	298 (25)	3	223.5	1.0 (0.26–2.7)	13.4 (3.4–36.5)
West and Middle Africa	113 (9)	2	75.4	1.8 (0.30–5.7)	26.5 (4.5–87.6)
East Africa	140 (12)	1	109.4	0.7 (0.036–3.5)	9.1 (0.46–45.1)
Southern Africa	67 (6)	0	57.0	0	0

*CI, confidence interval; NA, not applicable.
†Per 1,000 person-months.

previous travel to a dengue-endemic country, yellow fever vaccination for the current trip, and use of a mosquito net, the IRRs equaled 1.0 ($p>0.05$).

Recent DENV infection was positively correlated with fever, retro-orbital pain, myalgia, arthralgia, and skin rash (Table 4). Dengue-like illness was reported by 5 (36%; 95% CI 14%–62%) of 14 participants. Evidence for recent DENV infection was 7.3× more prevalent among those with than without dengue-like illness. No traveler reported symptoms signs or suggestive of dengue hemorrhagic fever.

Discussion

In this prospective study, the serology-based attack rate and incidence rate for recent DENV infection in short-term travelers to areas in which dengue is endemic were substantial: 1.2% and 14.6 per 1,000 person-months, respectively. Although most seroconversions occurred in travelers to Southeast and southern Asia, 3 occurred in travelers to sub-Saharan Africa.

The risk for recent infection was significantly related to travel during the rainy months, as others have reported (*10*). The use of insect repellent containing DEET appeared to be protective, although not significantly so. The presence of fever, retro-orbital pain, myalgia, arthralgia, and skin rash all had predictive value for recent infection. Dengue-like illness was the strongest predictor. Nevertheless, 64% of infections were asymptomatic.

For as many as 6.5% of travelers, serology results suggested previous DENV infection. This rate was strongly related to birth in a dengue-endemic country and a history of frequent travel to such countries. Increasing age and the travel purpose of visiting friends or relatives were also predictive for previous DENV infection, as described (*5*).

Studies based on febrile travelers returning from the tropics have reported dengue fever in ≈2% of those who sought medical help (*12*,*23*). Some studies have suggested that this proportion has increased in the past 2 decades (*3*,*8*,*9*,*24*); others showed no significant increase (*25*).

All these studies were retrospective. They were based on persons who sought medical attention after travel and thereby missed persons who never had symptoms, whose symptoms resolved during travel, or who never consulted a doctor. These studies were influenced by referral bias and relied only on posttravel serologic confirmation. They could not compare characteristics between patients and persons who remained well, and the studies lacked valid denominator data to determine absolute risk. Interpreting their findings is therefore difficult.

The best methodologic approach for estimating incidence rates of clinical and subclinical travel-related DENV infections is to follow a cohort of travelers prospectively (*26*). We found only 2 such studies. In 1 study, 6.7% of 104 travelers from Israel who spent on average 6.1 months (range 3–16 months) in Southeast Asia, South America, or Africa seroconverted (*11*), yielding an incidence rate of 11.0 per 1,000 person-months. This incidence rate is comparable to the rate found in our study, although our study differed in travelers' destination and duration of exposure and in test characteristics.

In the other prospective study, performed during 1991–1992, the seroconversion rate for 447 travelers from the Netherlands who spent a median of 4 weeks in Asia was 3.6%, resulting in an incidence rate of 36.9 per 1,000 person-months (*10*). In our study, the seroconversion and incidence rates in travelers to Southeast Asia were lower: 1.6% and 18.5 per 1,000 person-months, respectively. Our different findings may reflect different test characteristics and differences in exposure and risk behavior related to factors such as travel destination, year of exposure, and measures taken to prevent mosquito bites.

The incidence rate for persons in our study who traveled to sub-Saharan Africa appeared to be substantial

Table 4. Signs and symptoms among travelers with seroconversion in antibody level for dengue virus in 1,207 persons who traveled from the Netherlands to dengue-endemic countries, October 1, 2006–September 30, 2007*

Sign or symptom	Total no.	Recent DENV, no. (PR, %)	PRR (95% CI)	p value
Fever, temperature ≥38°C	113	5 (4.4)	5.6 (1.8–17.0)	**0.002**
No fever	1,094	9 (0.8)	1	
Headache	398	5 (1.3)	1.1 (0.38–3.4)	0.83
No headache	809	9 (1.1)	1	
Retro-orbital pain	100	4 (4.0)	4.6 (1.4–14.8)	**0.011**
No retro-orbital pain	1,107	10 (0.9)	1	
Myalgia	166	5 (3.0)	3.6 (1.2–10.8)	**0.024**
No myalgia	1,041	9 (0.9)	1	
Arthralgia	72	3 (4.2)	4.4 (1.2–16.3)	**0.025**
No arthralgia	1,135	11 (1.0)	1	
Rash	105	5 (4.8)	6.1 (2.0–18.5)	**0.001**
No rash	1,102	9 (0.8)	1	
Diarrhea	581	10 (1.7)	2.7 (0.85–8.7)	0.092
No diarrhea	626	4 (0.6)	1	
Dengue-like illness†	89	5 (5.6)	7.3 (2.4–22.4)	**<0.0001**
No dengue-like illness	1,118	9 (0.8)	1	

*DENV, dengue virus infection; PR, prevalence rate; PRR, prevalence rate ratio; CI, confidence intervals. **Boldface** indicates significance (p<0.5).
†Fever (temperature ≥38°C) with ≥1 of the following: myalgia, arthralgia, headache, retro-orbital pain, or skin rash.

(13.4/1,000 person-months), although the 95% CI was wide (3.4–36.5), and cross-reactions with other flaviviruses or previous yellow fever vaccination cannot be excluded. However, cross-reactions with yellow fever vaccination are unlikely because the travelers to Africa who seroconverted for anti-DENV either did not receive a yellow fever vaccination during the study period (2 travelers) or had dengue-like illness (1) (Table 3). These travelers visited Senegal and the Gambia, Mali and Burkina Faso, and Madagascar. All these countries reported dengue cases during the study period (*14*,*27*,*28*). According to data from the GeoSentinel Network (*12*) and the TropNetEurope Network (*5*), only a small proportion (5%–8%) of clinical dengue cases in travelers are acquired in Africa. However, distributions yielded by these data reflect not only global dengue activity but also the popularity of tourist destinations and physicians' expectations about dengue endemicity and their inclination to test for the disease. Dengue endemicity in Africa may have changed, and the risk for travelers may thus be higher than expected (*27*). More studies are needed.

The ratio of apparent to unapparent infection in our study was 1:1.8. The 2 other prospective studies reported ratios of 1:0.75 (*11*) and 1:3 (*10*). Variations in these ratios may reflect variation in strain virulence, the influence of initial viral load, or host factors such as susceptibility.

Our study has some limitations. First, our sample size was too small to yield a precise estimate of disease incidence per region or per destination. For example, travelers to Southeast Asia are considered to be at higher risk than travelers to other regions (*5*,*12*). This finding could not be confirmed in our study. Second, selection bias may have occurred. Although participants in our study are comparable to the average traveler (*29*), they were all seeking pretravel health advice. Thus, they perhaps had a higher than average health awareness, particularly after receiving oral and written travel advice, learning about the study, and agreeing to participate. Nevertheless, their average use of insect repellent containing DEET was only 45% of travel days, and 17% of participants used no repellent at all. Third, the risk for travel-related DENV infection also can depend on endemicity and on outbreaks in a particular country during a particular time of travel. Also, among travelers, DENV transmission has annual oscillations (*12*,*30*). Furthermore, sometimes epidemics occur in tourist areas, other times in areas never reached by tourists. Although 2006–2007 were not years with high endemicity for most dengue-endemic countries, the exact contribution of these factors on our findings is unknown.

A final limitation is that, although the serologic tests used are highly sensitive and specific, false-negative and false-positive test results may have occurred. For example, 7 travelers seroconverted only for anti-DENV IgG and not for anti-DENV IgM. Although this may be because of false-positive IgG or false-negative IgM test results, these results may also suggest secondary infection. Unfortunately, no test characteristics are available that apply exactly to our study design; the best available test characteristics are probably those from studies that used paired serum samples (*6*,*17*,*18*,*20*). With test specificity of ≤96%, ≥4% of positive samples actually give false-positive results. Subclinical infections with flaviviruses other than DENV may account for some of the seroconversions (*31*). Also, cross-reactions can affect the interpretation of test results in travelers who have received a flavivirus vaccine. In 1 study, 2 (15%) of 13 persons vaccinated against yellow fever had positive test results for DENV-IgG 1–2 months after the vaccination (*31*). In our study, however, recent and previous DENV infections were not related to yellow fever

vaccination. False-positive test results can also occur just by chance. Because the serologic tests used were validated in a clinical environment among patients suspected to have dengue, their positive predictive value will be lower when applied as a screening tool in a more general sample of travelers in which disease occurrence is lower.

Unfortunately, no true standard exists for confirming or ruling out DENV infections. Even a plaque-reduction neutralization test can cross-react (*32*), and PCR is useful only in the very early stage of dengue disease (*33*).

In conclusion, although valid data regarding the actual frequency of DENV infection in international travelers is elusive, our prospective study confirms that the incidence rate for recent DENV infection in short-term travelers to areas in which dengue is endemic is substantial. We found no differences in incidence rate compared with other serology-based prospective cohort studies performed in the 1990s. Thus, the increase in annually reported dengue cases from network studies is possibly related to the increase in international travel and the expansion of DENV and its vector to new areas rather than to an increase in DENV transmission. Until an effective and safe vaccine that provides effective, long-term immunity against all 4 serotypes of DENV has been developed, the only useful preventive measure for travelers to areas where dengue is endemic is to avoid mosquito bites, particularly during the rainy season.

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Experimental Infection of *Amblyomma aureolatum* Ticks with *Rickettsia rickettsii*

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We experimentally infected *Amblyomma aureolatum* ticks with the bacterium *Rickettsia rickettsii*, the etiologic agent of Rocky Mountain spotted fever (RMSF). These ticks are a vector for RMSF in Brazil. *R. rickettsii* was efficiently conserved by both transstadial maintenance and vertical (transovarial) transmission to 100% of the ticks through 4 laboratory generations. However, lower reproductive performance and survival of infected females was attributed to *R. rickettsii* infection. Therefore, because of the high susceptibility of *A. aureolatum* ticks to *R. rickettsii* infection, the deleterious effect that the bacterium causes in these ticks may contribute to the low infection rates (<1%) usually reported among field populations of *A. aureolatum* ticks in RMSF-endemic areas of Brazil. Because the number of infected ticks would gradually decrease after each generation, it seems unlikely that *A. aureolatum* ticks could sustain *R. rickettsii* infection over multiple successive generations solely by vertical transmission.

The bacterium *Rickettsia rickettsii* is the etiologic agent of the deadliest rickettsiosis, Rocky Mountain spotted fever (RMSF), which is referred to as Brazilian spotted fever in Brazil (*1*). The distribution of *R. rickettsii* is restricted to the Americas; confirmed cases of RMSF have been reported in Canada, United States, Mexico, Costa Rica, Panama, Colombia, Brazil, and Argentina. Different tick species have been implicated as vectors of *R. rickettsii* in different geographic areas. Whereas the ticks *Dermacentor andersoni* and *D. variablilis* are the main vectors in the United States, the *Amblyomma cajennense* tick is the most common vector in South America (*1*,*2*). The tick *Rhipicephalus sanguineus* has also been implicated as a vector for *R. rickettsii* in a few areas in Mexico and the state of Arizona in the United States (*2*,*3*). However, *A. aureolatum* ticks are the main vector in the metropolitan area of São Paulo, Brazil, distinguishing this area from the remaining RMSF-endemic areas of Latin America (*1*,*4*,*5*).

Adult *A. aureolatum* ticks feed chiefly on Carnivora species (mostly domestic dogs), but immature ticks (larvae, nymphs) prefer to feed on passerine birds and a few rodent species (*6*). Humans have reported being attacked only by adults, usually by a single tick (*7*), because population density of *A. aureolatum* ticks is usually low (*8*). One field study in an RMSF-endemic area of São Paulo found that <1% of the *A. aureolatum* adult ticks were infected by *R. rickettsii* (*5*). The reasons for such a low infection rate are not clear; a recent laboratory study reported that up to 100% of *A. aureolatum* larvae efficiently acquired and maintained the *R. rickettsii* infection to the nymphal stage, after a larval feeding on experimentally infected guinea pigs (*9*). Therefore, we evaluated the transstadial maintenance and transovarial transmission of *R. rickettsii* in *A. aureolatum* ticks through 4 consecutive generations of this tick in the laboratory; the vector competence of larvae, nymphs, and adults; and deleterious effects of *R. rickettsii* on the survival of larvae and nymphs and on the reproductive performance of female ticks.

Materials and Methods

In a laboratory experiment previously reported (*9*), the first generation (F_1) larval progeny of engorged *A. aureolatum* female ticks collected in Atibaia, São Paulo State, Brazil, were allowed to feed on 4 *R. rickettsii*–infected guinea pigs (infected group) and 2 uninfected guinea

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pigs (control group). These guinea pigs were infected by intraperitoneal inoculation of a homogenate of *R. rickettsii*–infected guinea pig organs, as detailed previously (*9*). The Taiaçu strain of *R. rickettsii* used in this experiment was the first guinea pig passage from a naturally infected *A. aureolatum* tick, which was cryopreserved before this strain was adapted for in vitro growth (*5*). Engorged tick larvae obtained from the infected and control groups of guinea pigs were held in an incubator at 23°C and 90% relative humidity for molting to nymphs. As reported (*9*), 100% of the resultant nymphs from the infected group were shown to be infected by *R. rickettsii,* whereas no nymph from the control group was infected by rickettsiae. These F_1 nymphs were simultaneously used for the study we report here.

The F_1 unfed nymphs of infected and control groups were reared separately in the laboratory for 4 consecutive generations until they were F_4 unfed adults. Throughout the experiment, infestations with infected ticks and control groups were done in parallel; infested animals were held in the same room under the same environmental conditions as the controls. Male guinea pigs and female rabbits were infested with larvae and nymphs, and dogs and rabbits were infested with adult ticks. However, in each infestation with a given tick stage, different individuals of the same host species were used at the same time for infected and control groups. Every guinea pig or rabbit used in this study was tick naive; these animals were provided by a private laboratory that raised the animals under proper sanitary conditions. The dogs used for adult infestations had been infested by *A. aureolatum* ticks in a previous study (*10*), when they were also infected with *R. rickettsii*; the dogs were therefore immune to RMSF. Infestation of each animal was performed inside a feeding chamber glued to its shaved dorsum, as previously described (*11*). All infested animals had their temperature rectally measured daily from the day of infestation (day 0) to 21 days postinfestation. When present, skin lesions (e.g., scrotal reactions) during this period were also noted. Naturally detached engorged larvae, nymphs, or female ticks were recovered daily from the feeding chambers of the infested animals of both groups and immediately taken to a single incubator adjusted to 23°C and 95% relative humidity for molting (for engorged larvae and nymphs) or for egg laying and incubation (for engorged females). Engorged females had their individual weight measured the day they detached from the host. In addition, the total egg mass deposited by each female was weighed on the day of the end of oviposition and a conversion efficiency index (CEI = mg egg mass/mg engorged female × 100), which measures the efficiency with which a female tick converts body weight into eggs (*12*), was determined for each female that oviposited. Percentage of egg hatching for each egg mass was visually estimated (*13*).

During the experiment, random samples of unfed F_1 nymphs and adults; F_2 eggs and nymphs; and F_4 eggs, larvae, nymphs, and adults from both infected and control groups were submitted for DNA extraction as described (*9*) and tested by PCR targeting a 401-bp fragment of the rickettsial *gltA* gene, as described (*9*). Eggs were tested in pools (1 egg pool containing 5–10 eggs/female), whereas larvae, nymphs, and adults were tested individually. At the end of the study, PCR products from 3 F_4 nymphs underwent DNA sequencing (*5*), and the resultant sequence was compared with GenBank data by BLAST analysis (www.ncbi.nlm.nih.gov/blast).

All tick-infested guinea pigs and rabbits were tested for seroconversion to *R. rickettsii* antigens. For this purpose, blood samples were collected at 0 days postinfestation and 21 days postinfestation; these samples were tested for anti–*R. rickettsii* reactive antibodies by immunofluorescence assay (IFA), as previously described (*14*). Some infested guinea pigs that died before the 21 days postinfestation were not tested by IFA because a second blood sample was not obtained; however, a fragment of their lungs was submitted to DNA extraction by using the DNeasy Tissue Kit (QIAGEN, Chatsworth, CA, USA) and tested by the same PCR protocol referenced above.

During the experiment, tick biologic parameters were compared between infected and control groups. For this purpose, larval and nymphal molting success and female oviposition success (i.e., death of engorged ticks) were compared by the χ^2 test. In addition, weight of engorged females and their corresponding egg masses, percentage of egg hatching, and CEI values were compared by Student *t* test. Values were considered significantly different when $p<0.05$. The study was approved by the Bioethical Committee in Animal Research of the Faculty of Veterinary Medicine of the University of São Paulo (protocol no. 1644/2009).

Results

The infected tick group remained infected by rickettsiae through 4 consecutive generations, until the end of the experiment (F_4 unfed adults). In all infestations performed with ticks from this group, fever developed in all guinea pigs and rabbits 5–9 days postinfestation. Guinea pigs also had scrotal reactions characterized by swelling, followed by necrosis in most animals. Among the 16 guinea pigs infested with infected larvae or nymphs, 8 died during the febrile period. Of the 8 dead guinea pigs, 6 had their lungs tested by PCR, which detected rickettsial DNA. The remaining guinea pigs plus the rabbits showed seroconversion for *R. rickettsii* at 21 days postinfestation (Table 1). In contrast, no guinea pig or rabbit infested with ticks from the control group experienced fever or seroconverted; i.e., they were nonreactive for *R. rickettsii* at both 0 days postinfestation

Table 1. Clinical data of hosts infested by a *Rickettsia rickettsii*–infected colony of *Amblyomma aureolatum* ticks, and results of PCR performed on unfed ticks from 4 consecutive generations (F_1 to F_4)*

Tick stage and generation	Infested hosts				No. deaths	Diagnostic test		PCR of unfed ticks, no. infected/no. tested
	Host, no.	No. ticks/host	Fever† No.	Fever† Onset, dpi		PCR‡	IFA§	
Nymph–F_1	Guinea pig, 2	50	2	6	2	Pos	ND	45/45¶
Adult–F_1	Dog, 2	20 couples	0	–	0	ND	ND	10/10
Larva–F_2	Guinea pig, 2	1,000–2,000	2	7–8	1	ND	Pos	–
Larva–F_2	Rabbit, 3	1,000–2,000	3	7–9	0	ND	Pos	–
Nymph–F_2	Guinea pig, 3	80	3	5	2	Pos	Pos	30/30
Nymph–F_2	Rabbit, 1	200	1	6	0	ND	Pos	–
Adult–F_2	Rabbit, 2	7 couples	2	7–8	0	ND	Pos	–
Larva–-F_3	Guinea pig, 4	1,000–2,000	4	7–8	2	Pos	Pos	–
Nymph–F_3	Rabbit, 1	400	1	6	0	ND	Pos	–
Adult–F_3	Dog, 1	12 couples	0	–	0	ND	ND	–
Larva–F_4	Guinea pig, 5	1,000–3,000	5	6–7	1	ND	Pos	100/100
Nymph–F_4	Rabbit, 1	500	1	5	0	ND	Pos	30/30

*dpi, days postinfestation; IFA, immunofluorescence assay; pos, positive; ND, not done; –, data not collected.
†Rectal temperature ≥40°C.
‡PCR performed on lung-extracted DNA from guinea pigs that died during the febrile period.
§A positive result means seroconversion, namely nonreactive (titer <64) at 0 DPI, and reactive (titer >1,024) at 21 dpi.
¶Previously reported by Labruna et al. (*9*).

and 21 days postinfestation. Fever did not develop in the dogs infested with generations F_1 and F_3 adult ticks, and serum was not tested on 0 days postinfestation and 21 days postinfestation because it was already known that the dogs were seroreactive to *R. rickettsii* because they had been previously infected in another study (*10*).

All PCRs performed on unfed ticks from the infected group resulted in amplicons compatible with *R. rickettsii*. These amplicons resulted from 45 F_1 nymphs, 10 F_1 adults (5 males, 5 females), 30 F_2 nymphs, 100 F_4 larvae, 30 F_4 nymphs, and 6 F_4 adults (3 males, 3 females), which were all tested individually (Table 1). In addition, 12 F_2 egg pools and 5 F_4 egg pools, derived from all F_1 and F_3 infected engorged females, also yielded PCR amplicons compatible with *R. rickettsii*. The 100 PCR-positive F_4 larvae cited above encompassed 5 groups of 20 larvae, each derived from a different F_3 engorged female. PCR products from 3 F_4 nymphs underwent DNA sequencing, and the resultant sequence was 100% identical to *R. rickettsii* strain Taiaçu, available in GenBank (accession no. DQ115890).

For the control group, no visible amplicon was generated by all ticks tested by PCR, which included 20 F_1 nymphs, 6 F_1 adults (3 males, 3 females), 10 F_2 nymphs, 30 F_4 larvae, 20 F_4 nymphs, and 6 F_4 adults (3 males, 3 females), which were all tested individually, and 13 F_2 egg pools and 6 F_4 egg pools, derived from all F_1 and F_3 control engorged females. The 30 PCR-negative F_4 larvae cited above encompassed 3 groups of 10 larvae, with each group derived from a different F_3 engorged female.

Although the mortality rate for engorged F_4 larvae and F_3 nymphs was higher for the infected group than the control group, overall molting success of engorged larvae and nymphs was similar between infected and control ticks because there was no significant difference in the molting success of these ticks when the 4 generations were grouped. However, there was an overall lower egg-laying success of engorged females from the infected group when compared with the control group (Table 2); i.e., although 89.7% of the control engorged females successfully oviposited in the incubator, only 66.7% of the infected females oviposited in the same incubator. Regarding the reproductive performance of these females, a few significant differences were observed between infected and control females, generally in favor of control ticks (online Appendix Table, www.cdc.gov/EID/content/17/5/829-appT.htm).

Discussion

In the study reported here, *R. rickettsii* was preserved by transstadial maintenance and transovarial transmission in *A. aureolatum* ticks for 4 consecutive generations, because all tested eggs, larvae, nymphs, and adults from the infected group were shown by PCR to contain rickettsial DNA, from the first to the fourth generation. In addition, infestations of guinea pigs and rabbits with larvae and nymphs from the 4 generations and adults from the third generation confirmed that ticks of these 3 developmental stages from the 4 generations were infected by *R. rickettsii* because all infested guinea pigs and rabbits became infected by *R. rickettsii,* which was confirmed by seroconversion through IFA or PCR in addition to compatible clinical data. These results also confirm that larvae, nymphs, and adults of *A. aureolatum* ticks are competent vectors of *R. rickettsii*. Together, our results strongly support clinical and epidemiologic data that have implicated the *A. aureolatum* tick as the main vector of *R. rickettsii* in the metropolitan area of São Paulo (*4*,*5*,*9*,*15*).

Table 2. Molting and oviposition success of *Amblyomma aureolatum* ticks infected by *Rickettsia rickettsii* (infected group) and noninfected (control group) through 4 consecutive generations (F_1 to F_4) in the laboratory*

Tick generation	No. larvae that molted to nymphs/ no. recovered engorged larvae (% molting success)		No. nymphs that molted to adults/ no. recovered engorged nymphs (% molting success)		No. females that oviposited/ no. recovered engorged females (% oviposition success)	
	Infected	Control	Infected	Control	Infected	Control
F_1	632/679 (93.1)	226/250 (90.4)	68/71 (95.8)	25/29 (86.2)	12/16 (75)	13/13 (100)
F_2	300/349 (86.0)	791/959 (82.5)	57/60 (95.0)	24/28 (85.7)	5/10 (50)	7/9 (77.8)
F_3	292/443 (65.9)	737/1,179 (62.5)	49/110 (44.5)	51/68 (75.0)†	5/7 (71.4)	6/7 (85.7)
F_4	721/1,278 (56.4)	868/1,358 (63.9)†	138/148 (93.2)	46/52 (88.5)	–	–
Total	1,945/2,749 (70.7)	2,622/3,746 (70.0)	312/389 (80.2)	146/177 (82.5)	22/33 (66.7)	26/29 (89.7)†

*–, data not collected.
†Molting or oviposition success values for infected and control ticks of the same tick stage were significantly different ($p<0.05$).

All infected egg pools tested by PCR yielded rickettsial DNA, indicating a transovarial transmission rate (the proportion of infected females giving rise to at least 1 infected egg or larva) of 100% among *R. rickettsii*–infected females. Because all the individual larvae from the infected group tested by PCR also yielded rickettsial DNA, a filial infection rate (proportion of infected eggs or larvae obtained from an infected female) of 100% is also likely to have occurred. In contrast, no egg pool or individual larva from the control group yielded rickettsial DNA by PCR. These results are in agreement with the larval infestations, which resulted in confirmed rickettsiosis in all guinea pigs or rabbits infested by larvae from the infected group and with no rickettsiosis in animals infested with control group larvae. Although transovarial transmission of spotted fever rickettsiae in ticks seems to occur worldwide (*16*), including *R. rickettsii* in New World ticks (*17*–*23*), few studies have quantified this perpetuation route. Previous studies in the United States demonstrated a 100% transovarial transmission rate and a 100% filial infection rate of *R. rickettsii* in *D. variabilis* naturally infected female ticks and in *D. andersoni* female ticks, either naturally infected or experimentally infected during the larval stage (*20*,*24*).

In the study reported here, although F_2 female ticks fed on susceptible rabbits, F_1 and F_3 females fed on dogs previously infected by *R. rickettsii*. As expected, these dogs showed no clinical signs during the study, a condition certainly related to their immune status because they were shown to have high antibody titers against *R. rickettsii* (data not shown). Regardless of feeding on immune (dogs) or susceptible (rabbits) hosts, 100% transovarial transmission rates and filial infection rates were found for all generations evaluated. Similarly, Burgdorfer and Brinton (*24*) observed 100% transovarial transmission rates and filial infection rates for *D. andersoni* female ticks that fed on either immune or susceptible hosts, resulting in no alteration of biological characteristics of the bacterium. Thus, the host immune status to *R. rickettsii* does not seem to alter the transovarial transmission of *R. rickettsii* in ticks.

Overall, no expressive differences in mortality rates were observed between engorged immature ticks from the infected and control groups. These results contrast with those of a previous study (*21*) that reported much higher mortality rates for infected *D. andersoni* engorged larvae and nymphs (34.9%–97.7%) than those observed for uninfected sibling ticks (1.4%–2.1%) and also with those of experimental studies on *R. conorii* in *R. sanguineus* ticks, in which significant mortality rates of immature ticks were observed when compared with uninfected ticks (*25*,*26*). On the other hand, our results demonstrated that the mortality of *R. rickettsii*–infected engorged females was 3× higher than the mortality of uninfected females; i.e., oviposition success was only 66.7% in the former and 89.7% in the latter. In addition, the reproductive performance of uninfected females was significantly higher than that of infected females, as demonstrated by higher egg mass weight for control ticks. Because both the infected and control ticks were siblings derived from the same field-engorged females used to start a laboratory colony, reared under the same laboratory conditions during the whole study, we conclude that the lower survival and reproductive performance of infected females was a result of a deleterious effect caused by the *R. rickettsii* infection. These results are in agreement with those of a previous study (*24*), which reported higher mortality of *R. rickettsii*–infected *D. andersoni* and *D. variabilis* engorged female ticks and lower egg masses oviposited by these females than by uninfected females. This higher mortality and lower egg mass was attributed to massive rickettsial development in the female body, including the ovaries (*24*). Tick mortality is much more influential on the tick population when it occurs in engorged females; i.e., although each dead egg, larva, or nymph is only less 1 subsequent larva, nymph, or adult, respectively, in the tick population, a dead engorged female represents thousands of eggs fewer in the following generation.

Different field studies in Brazil and in the United States have reported that <1% of the *Dermacentor* spp. and *A. aureolatum* ticks, respectively, are found naturally infected by *R. rickettsii* within RMSF-endemic

areas (*1*,*5*,*27*). On the other hand, the present study demonstrated that besides being highly susceptible to *R. rickettsii* infection, *A. aureolatum* ticks are highly efficient in maintaining the infection through 100% transstadial transmission, transovarial transmission, and filial infection rates. The main reason for these contrasting findings is the deleterious effect the *R. rickettsii* infection causes in ticks, as previously demonstrated for *Dermacentor* spp. ticks (*21*,*24*,*27*). Therefore, despite of the high susceptibility of *A. aureolatum* ticks to *R. rickettsii* infection, the higher mortality and reduced reproductive performance of infected engorged females may contribute to low infection rates among *A. aureolatum* tick field populations in RMSF-endemic areas of the São Paulo metropolitan area, such as the 0.9% infection rate previously reported (*5*).

On the basis of our results, it seems unlikely that *A. aureolatum* could sustain *R. rickettsii* infection over multiple successive generations solely by vertical transmission because the number of infected ticks would gradually decrease after each generation. Thus, horizontal transmission through the participation of amplifier vertebrate hosts in the formation of new lineages of infected ticks seems to be crucial for maintenance of *R. rickettsii* in the RMSF-endemic areas where the *A. aureolatum* tick is implicated as the principal vector, just as has been reported for RMSF-endemic areas in the United States, where *Dermacentor* spp. ticks are the vector (*21*,*27*). Although natural amplifier hosts are known for *Dermacentor* spp. ticks in RMSF-endemic areas in the United States (*27*), and for *A. cajennense* ticks in areas endemic for Brazilian spotted fever in Brazil (*1*), they are not known for *A. aureolatum* ticks. Further studies should test the natural hosts of *A. aureolatum* immature tick stages, which are some passerine birds and small rodents (*6*), for their competence to act as amplifier hosts of *R. rickettsii* to *A. aureolatum* ticks. Regarding dogs, the main host for the adult stage of *A. aureolatum* ticks within the São Paulo metropolitan area, even though it has been shown that dogs are capable of having rickettsial infections sufficient to infect other tick species (i.e., they are a competent amplifier host) (*23*), only adult-stage *A. aureolatum* ticks feed on dogs. Because transovarial transmission rates are likely to be low or absent when the primary infection of ticks occurs during adult feeding (*18*,*23*,*24*), the epidemiologic influence of dogs as amplifier hosts of *R. rickettsii* for *A. aureolatum* ticks is questionable. Therefore, future studies should target potential hosts of the immature stages of *A. aureolatum* ticks that could act as amplifier hosts.

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Genotypic Profile of *Streptococcus suis* Serotype 2 and Clinical Features of Infection in Humans, Thailand

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To examine associations between clinical features of *Streptococcus suis* serotype 2 infections in humans in Thailand and genotypic profiles of isolates, we conducted a retrospective study during 2006–2008. Of 165 patients for whom bacterial cultures of blood, cerebrospinal fluid, or both were positive for *S. suis* serotype 2, the major multilocus sequence types (STs) found were ST1 (62.4%) and ST104 (25.5%); the latter is unique to Thailand. Clinical features were examined for 158 patients. Infections were sporadic; case-fatality rate for adults was 9.5%, primarily in northern Thailand. Disease incidence peaked during the rainy season. Disease was classified as meningitis (58.9%) or nonmeningitis (41.1%, and included sepsis [35.4%] and others [5.7%]). Although ST1 strains were significantly associated with the meningitis category (p<0.0001), ST104 strains were significantly associated with the nonmeningitis category (p<0.0001). The ST1 and ST104 strains are capable of causing sepsis, but only the ST1 strains commonly cause meningitis.

Streptococcus suis, an emerging zoonotic pathogen, causes invasive infections in persons who are in close contact with infected pigs or contaminated pork-derived products (*1*). On the basis of capsular polysaccharides, 33 serotypes of *S. suis* have now been identified. Of these, serotype 2 is the most prevalent type in humans infected with this pathogen (*1*,*2*). Since the largest outbreak of human *S. suis* infection in 2005, in Sichuan Province, People's Republic of China (*3*), this disease has been increasingly recognized worldwide. The numbers of reported cases, especially in persons from Southeast Asian countries, have increased dramatically during past few years (*4*).

In Thailand, at least 300 cases of *S. suis* infection in humans have been reported (*5*–*11*). Although an outbreak of *S. suis* infections was confirmed in Phayao Province during May 2007 (*9*), most cases in humans occur sporadically and are primarily located in the northern region of this country (*6*–*11*). A relatively low incidence of cases with *S. suis* serotype 14 has also been reported in this region (*12*). Although previous studies have reported high frequencies (59.0%–88.7%) of *S. suis* infections in persons in this area who ate raw pork products (*8*–*11*), the pathogenesis of this disease, including routes of transmission, is unclear.

The major clinical manifestations of the disease are bacterial meningitis and sepsis, but other manifestations have been reported (*1*,*4*, *8*,*10*,*13*). Most cases of bacterial meningitis can be attributed to the hematogenic spread of invasive bacteria, but how circulating bacteria cross the blood–cerebrospinal fluid (CSF) barrier and cause meningitis is not clear (*14*,*15*). Furthermore, the overall clinical features of this disease have not been extensively and comprehensively investigated in Southeast Asian countries.

A variety of virulence factors associated with *S. suis* have been reported (*16*–*20*), but none have been proven to be essential for the host defense of this disease, except

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the capsular polysaccharide (*19*). In serotype 2 isolates obtained during a previous outbreak in Sichuan, China, an ≈89-kb DNA fragment, which has been associated with a pathogenicity island (89K PAI), was identified (*21*). The 89K PAI fragment encodes a 2-compartment signal transduction system, SalK-SalR, which is required for full virulence (*22*).

We report the results of a retrospective study of the clinical features of 158 cases of human infection with *S. suis* serotype 2 and the molecular epidemiology of 165 *S. suis* serotype 2 isolates. The study objective was to demonstrate associations between the clinical features of disease caused by *S. suis* serotype 2 in persons in Thailand and the genotypic profiles of the isolates. The study was reviewed and approved by the Ethics Committees of Research Institute for Microbial Diseases, Osaka University, and conducted according to the principles expressed in the Declaration of Helsinki.

Methods

Isolate Identification

From January 2006 through August 2008, a total of 1,154 unidentified streptococcal isolates from blood or CSF were collected from hospitals in all 76 provinces of Thailand. Biochemical testing of these isolates, using API Strep (bioMérieux, Durham, NC, USA) and *S. suis*–specific and *S. suis* serotype 2– or 1/2–specific PCR (*12*,*23*), confirmed 165 isolates from 34 hospitals in 25 provinces as *S. suis*. The final serotype of all strains was confirmed by coagglutination tests that used rabbit antiserum (Statens Serum Institute, Copenhagen, Denmark).

Genotypic Profiles of Isolates

Multilocus sequence type (MLST) testing was performed as described by King et al. (*24*), with a modification for *mutS* as described by Rehm et al (*25*). MLST alleles and the resulting sequence type (ST) were assigned by using the *S. suis* MLST database (http://ssuis.mlst.net). eBURST was used to identify the clonal complexes for these 165 serotype 2 strains within *S. suis*, and the overall structure of the population was obtained through the MLST database (*26*). Virulence-associated genes (VAG), including extracellular released protein factor (*epf*), muramidase-released protein (*mrp*), and suilysin (*sly*), and variants of *mrp* or *epf* were determined by PCR as described by Silva et al. (27), with minor modifications. Presence of the 89K PAI fragment was determined by PCR as reported by Chen et al (*21*). Pulsed-field gel electrophoresis (PFGE) was performed as described (*28*), and the pulsotypes were assigned to clusters of isolates with >80% similarity from the dendrogram. The dendrogram representing the genetic relationships between the representative pulsotypes from 165 *S. suis* serotype 2 strains was drawn by using the Cluster 3.0 software program and examined by using the TreeView program as described (*12*,*29*).

Clinical Features of Cases

Of the 165 patients whose culture results were positive for *S. suis* serotype 2, medical records for 158 were retrospectively reviewed by physicians at local hospitals in Thailand. Medical records for the remaining 7 patients were not available. The clinical manifestations were mostly divided into 2 categories: meningitis and nonmeningitis. The meningitis category involved confirmed meningitis, bacteremic meningitis, and probable meningitis. All patients in the meningitis category had typical meningeal signs, such as neck stiffness, and acute disease onset. Although bacteremic meningitis was defined as a case in which both CSF and blood cultures were positive, confirmed meningitis was defined as a case with a positive CSF culture only, and probable meningitis was defined as a case with a positive blood culture only. The nonmeningitis category included the clinical manifestations of sepsis and sepsis with focal signs other than meningitis (septic arthritis or spondylodiscitis, infective endocarditis, and bacteremic pneumonia). Sepsis was defined as systemic inflammatory response syndrome and a positive blood culture (*30*), and septic arthritis or septic spondylodiscitis was defined as described (*31*). Diagnosis of infectious endocarditis was based on the Duke criteria (*32*). Septic shock was also defined as described (*33*).

Statistical Analyses

Comparisons of the clinical characteristics between fatal and nonfatal cases were analyzed by using the χ^2 test or Fisher exact test with Stata version 10.0 software (StataCorp, College Station, TX, USA). Patient ages and periods of hospital admission were tested for normality of the distribution using the Kolmogorov-Smirnov test and were compared by using the Student *t* test with SPSS version 11.0 software (SPSS Inc., Chicago, IL, USA). Data were considered significant at $p<0.05$.

Results

Genotypic Profiles of Isolates

Of the 165 *S. suis* serotype 2 isolates, 123 were isolated from blood and 42 from CSF. eBURST analysis based on MLST enabled classification of these strains into 4 ST complexes: the ST1, ST27, ST29, and ST104 complexes (Table 1). ST126, a novel ST, has a single locus variant from ST1. The largest cluster of 89K PAI–carrying strains was ST1 (n = 81, 49.1%), which had the *epf*+/*sly*+/*mrp*+ genotype; these strains were isolated from blood and CSF. Another large cluster of non-89K PAI–carrying strains was

Table 1. Genotypic profiles of 165 clinical isolates of *Streptococcus suis* serotype 2, Thailand, January 2006–August 2008*

ST complex	ST	VAG†	Isolation site	89K PAI +	89K PAI –	No. (%) strains
1	1	*epf*–/*sly*+/*mrp*+	Blood	1	0	103 (62.4)
		epf+/*sly*+/*mrp*+	Blood	52	13	
			CSF	29	5	
		epf+/*sly*+/*mrp*^{s}	Blood	0	1	
			CSF	0	2	
	126	*epf*+/*sly*+/*mrp*+	Blood	1	0	3 (1.8)
			CSF	2	0	
27	28	*epf*–/*sly*–/*mrp*+	Blood	0	1	3 (1.8)
			CSF	0	2	
29	25	*epf*–/*sly*–/*mrp**	Blood	8	0	11 (6.7)
		epf–/*sly*–/*mrp*+	Blood	3	0	
	103	*epf*–/*sly*–/*mrp**	Blood	2	0	3 (1.8)
		epf–/*sly*–/*mrp*+	Blood	1	0	
104	104	*epf*–/*sly*+/*mrp*–	Blood	3	38	42 (25.5)
			CSF	0	1	
Total no. strains	NA	NA	NA	102	63	165 (100)

*ST, sequence type; VAG, virulence-associated gene; 89K PAI, an ≈89-kb pathogenicity island; CSF, cerebrospinal fluid; NA, not applicable.
†*mrp*^{s} and *mrp** are *mrp* variants that produce ≈750-bp and ≈1,800-bp fragments, respectively, by PCR (*23*,*34*).

ST104, which had the *epf*–/*sly*+/*mrp*– genotype (n = 39, 23.6%); most of these strains (n = 38) were isolated only from blood. ST103, ST104, and ST126 were found only in isolates from humans in Thailand.

PFGE of Isolates

Of the 165 serotype 2 strains, PFGE analyses identified 20 pulsotypes (Figure 1, panel A). Analysis of the dendrogram for these 20 pulsotypes revealed at least 16 clusters (I to XVI) (Figure 1, panel B). Although 5 pulsotypes of A were identified for the ST1 and ST126 strains, 2 major pulsotypes (A [n = 32] and A1 [n = 43]), A1 (n = 43), and A4 (n = 3) were grouped in 1 cluster. Pulsotype A2 (n = 21), which consisted of ST1 strains lacking the 89K PAI fragment, was classified into a distinguished cluster. PFGE showed diverse DNA patterns for strains ST25 and ST103. ST25 strains were classified into 5 clusters of I, II, III, IV, and VIII. ST103 strains were classified into 3 clusters of VI, XIV, and XV. Three ST28 strains lacking 89K PAI exhibited the unique DNA pattern of pulsotype D; these were classified into cluster XVI. Although 4 pulsotypes (H, H1, H2, and H3) were identified for ST104 strains, 2 major pulsotypes (H [n = 29] and H1 [n = 11]) in ST104 strains were classified into cluster VII. Collectively, clusters X and XI for ST1 and ST126 strains and cluster VII for ST104 strains accounted for the major 3 clusters found for cases in Thailand.

Geographic and Seasonal Distribution

Of the 165 isolates, 136 (82.4%) were from the northern region, 19 (11.5%) from the central region, 7 (4.2%) from the northeast region, and 3 (1.8%) from the eastern region (Table 2; Figure 2, panel A). No strains were isolated from the southern region. The dates of isolation suggest that human cases occur more frequently during the rainy season, June–August of each year (Figure 2, panel B).

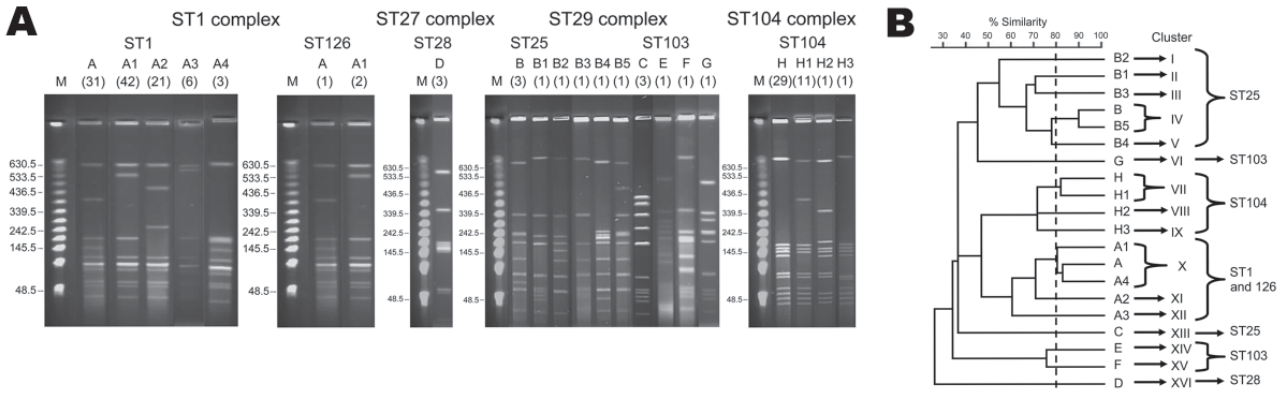

Figure 1. A) Pulsed-field gel electrophoresis profiles of 165 human isolates of *Streptococcus suis* serotype 2, after *Sma*I digestion. Numbers of isolates are indicated in parentheses below pulsotype numbers. B) Dendrogram generated from the pulsed-field gel electrophoresis profiles. ST, sequence type.

Table 2. Distribution of sequence types of 165 clinical isolates of *Streptococcus suis* serotype 2, by region, Thailand

Sequence type	North	Northeast	East	Central	South
1	85	6	1	11	0
25	11	0	0	0	0
28	3	0	0	0	0
103	1	0	1	1	0
104	33	1	1	7	0
126	3	0	0	0	0
Total	136	7	3	19	0

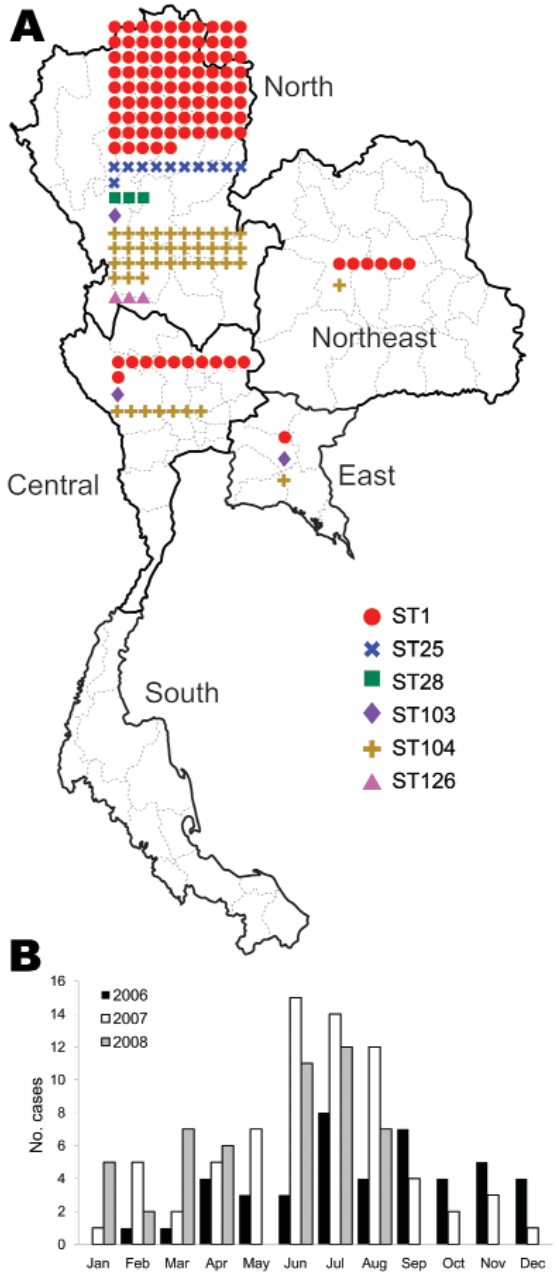

Figure 2. Distribution and sequence types (STs) of 165 human isolates of *Streptococcus suis* serotype 2, January 2006–August 2008, Thailand. A) Regions of isolation; B) monthly distribution of isolations.

Clinical Features of Cases

The clinical features of the 158 human cases of *S. suis* serotype 2 infection are summarized in Table 3. The median age (range) of the 155 patients for whom age was known was 55.0 (18–93) years; 72.8% were male. No cases in children were identified in this study. All 158 patients had been hospitalized; median duration (range) of hospitalization for the 158 patients was 11 (1–45) days; 15 (9.5%) patients died. No significant differences were found between the fatal and nonfatal cases with respect to patient age or period of admission.

The meningitis category (n = 93) included 22 cases of confirmed meningitis, 44 cases of bacteremic meningitis, and 27 cases of probable meningitis (Figure 3). The nonmeningitis category (n = 65) included sepsis with focal signs other than meningitis (n = 9) and sepsis (n = 56). Sepsis with focal signs other than meningitis included septic arthritis (n = 5), infective endocarditis (n = 3), and bacteremic pneumonia (n = 1). Of the 15 fatal cases, 8 were assigned to the meningitis category (probable meningitis [n = 6], meningitis [n = 1], bacteremic meningitis [n = 1]), 6 cases were sepsis, and 1 case was infective endocarditis (Table 3). Although the cases of bacteremic meningitis were significantly associated with a nonfatal outcome ($p = 0.043$), the probable meningitis cases were significantly associated with a fatal outcome ($p = 0.013$). The combined frequencies for the recent consumption of raw pork products and exposure to pigs were 39.9%. None of the clinical signs or possible risk factors, including recent exposure to pigs or raw pork products, or alcohol abuse, was significantly associated with a fatal outcome. Of the 158 patients, 154 parenterally received antimicrobial drugs, such as ceftriaxone, and data concerning antimicrobial drug treatment were not available for 4. Corticosteroids, such as dexamethasone, were used for only 4 patients.

Clinical Features and Genotype Profiles

The distributions of STs for the 158 human isolates for the meningitis and nonmeningitis categories are shown in Table 4. Although the ST1 strains were significantly associated with the meningitis category ($p<0.0001$), the ST104 strains were significantly associated with the nonmeningitis category ($p<0.0001$). The VAG profile of *epf+/sly+/mrp+*, which was dominant in the ST1 strains,

Table 3. Demographic and clinical features of 158 human cases of *Streptococcus suis* serotype 2 infections, Thailand, January 2006–August 2008*

Characteristic	All, n = 158	Fatal, n = 15; 9.5%	Nonfatal, n = 143; 90.5%	p value
Demographic				
Male sex, %	72.8	66.7	73.4	0.386
Mean (median) age, y†	56.6 (55.0)	53.9 (52.5)	57.0 (56.0)	0.264
Period of admission, d, mean (median)	12.5 (11)	10.1 (6)	12.9 (12)	0.737
Meningitis category, no. (%) cases				
Confirmed meningitis	22 (13.9)	1 (6.7)	21 (14.7)	0.348
Bacteremic meningitis	44 (27.8)	1 (6.7)	43 (30.1)‡	0.043
Probable meningitis	27 (17.1)	6 (40.0)	21 (14.7)§	0.013
Nonmeningitis category, no. (%) cases				
Septic arthritis	5 (3.2)	0	5 (3.2)	1
Infective endocarditis	3 (1.9)	1 (6.7)	2 (1.4)	0.905
Bacteremic pneumonia	1 (0.6)	0	1 (0.7)	1
Sepsis	56 (35.4)	6 (40.0)	50 (35.0)	0.698
Signs and symptoms, no. (%) cases				
Diarrhea	28 (17.1)	5 (33.3)	23 (16.1)	0.1
Hearing loss	34 (21.5)	4 (26.7)	30 (21.0)	0.409
Altered consciousness	35 (22.2)	4 (26.7)	31 (21.7)	0.434
Shock	9 (5.7)	2 (13.3)	7 (4.9)	0.205
Possible risk factors, no. (%) cases				
Recent consumption of raw pork products	52 (32.9)	5 (33.3)	47 (32.9)	0.589
Recent exposure to pigs	11 (7.0)	2 (13.3)	9 (6.3)	0.28
Alcohol abuse	33 (21.0)	5 (33.3)	28 (19.6)	0.178

*Statistical analyses were performed by using the χ^2 or Fisher exact test.
†Ages were not available for 3 patients.
‡One case of bacteremic meningitis was associated with pneumonia.
§Two cases of probable meningitis were associated with spondylodiscitis.

was also significantly associated with the meningitis category (p<0.0001). The VAG profile of *epf–/sly+/mrp–*, which was observed only in the ST104 strains, was also significantly associated with the nonmeningitis category (p<0.0001). Because the largest cluster of 89K PAI–carrying strains was associated with the VAG profile of *epf+/sly+/mrp+*, the presence of 89K PAI was also significantly associated with the meningitis category (p<0.0001). None of the genotypic profiles that included STs, VAG, and presence of 89K PAI were significantly associated with fatal or nonfatal outcomes (data not shown).

Discussion

Our finding that isolated *S. suis* serotype 2 strains peaked during the rainy season of 2006–2008 confirmed conclusions reached in previous small-scale studies

Table 4. Genotypic features of *Streptococcus suis* serotype 2 as risk factor for meningitis*

	Clinical category, no. (%) strains			
Feature	All, n = 158	Meningitis, n = 93	Nonmeningitis, n = 65	p value
Sequence type				
1	98 (62.0)	73 (78.5)	25 (38.5)	<0.0001†
104	40 (25.3)	6 (6.5)	34 (52.3)	<0.0001‡
25	11 (7.0)	7 (7.5)	4 (6.2)	0.478
28	3 (1.9)	2 (2.2)	1 (1.5)	0.632
103	3 (1.9)	2 (2.2)	1 (1.5)	0.655
126	3 (1.9)	2 (2.2)	0	0.201
VAG profile				
epf+/sly+/mrp+	97 (61.4)	72 (79.6)	25 (35.4)	<0.0001†
epf+/sly+/mrp^{s}	3 (25.3)	3 (3.2)	0 (0)	0.201
epf–/sly+/mrp–	40 (25.3)	6 (6.5)	34 (52.3)	<0.0001‡
*epf– /sly–/mrp**	10 (6.3)	6 (6.5)	4(6.2)	0.607
epf–/sly–/mrp+	7 (4.4)	5 (5.3)	2 (3.1)	0.392
epf–/sly+/mrp+	1 (1.0)	1 (1.1)	0 (0)	1
89K PAI profile, 89K PAI+	98 (62.0)	70 (75.3)	28 (43.1)	<0.0001†

*Statistical analyses were performed by using the χ^2 or Fisher exact test. VAG, virulence-associated gene; 89K PAI, ≈89-kb pathogenicity island.
†Significant association with the meningitis category.
‡Significant association with the nonmeningitis category.

conducted in northern Vietnam and Hong Kong (*35*,*36*). The predominant distribution of these isolates in northern Thailand is also in accordance with previous reports (*6*–*11*). However, why no human cases were identified in southern Thailand remains uncertain. A recent study from Hong Kong reported heavy contamination of *S. suis* in raw pork meat at local supermarkets or wet markets; therefore, a hot and humid climate may facilitate the growth of *S. suis* in raw pork products in those markets (*37*) and increase the risk for *S. suis* infections in humans in northern Thailand. The finding of no cases in children suggests that the routes of transmission are associated with adult behavior.

A recent study from northern Thailand, based on 20 human isolates collected during 1998–2002, reported that the most common isolates of *S. suis* serotype 2 were ST25 (40%), followed by ST1 (15%) and ST103 (15%) (*34*). By contrast, the MLST and PFGE results in this study clearly demonstrated that ST1 strains with major pulsotypes of A, A1 and A2, and ST104 with major pulsotypes of H and H1 were currently circulating in the same region of Thailand during 2006–2008. Collectively, these data suggest dynamic replacement of STs from ST25 to ST1 and ST104 among serotype 2 strains during recent years in this region.

Although *S. suis* serotype 2 has been reported to be the most frequent cause of bacterial meningitis in adults in Vietnam (*13*,*35*), other clinical manifestations, such as sepsis and infectious endocarditis, have also been found to be common in Thailand (*6*,*8*,*11*). Of the 158 human cases in the study reported here, ≈60% were assigned to the meningitis category and ≈35% were sepsis. Other clinical manifestations, including infective endocarditis, were rare. The findings reported here demonstrate significant associations between the ST1 strains and the meningitis category and between the ST104 strains and the nonmeningitis category. These findings indicate that both the ST1 and ST104 strains cause bacteremia and sepsis but that the ST1 strains are more likely to cross the blood–CSF barrier and subsequently result in meningitis. Because ≈80% of the cases in the meningitis category were caused by strains with ST1, as evidenced by a VAG profile of *epf*+/*sly*+/*mrp*+ and 89K PAI, these genotypic profiles of *S. suis* serotype 2 may favor bacterial survival and multiplication in the bloodstream, which would result in high levels of bacteremia, crossing of the blood–CSF barrier, and invasion of the meninges and the central nervous system (*15*). Our PFGE data showed that the pulsotype A1 found in serotype 2 strains with ST1 was identical to pulsotype 11 of serotype 2 strains with ST1 from Vietnam and pulsotype I of the serotype 2 strains with ST1 from Hong Kong (*13*,*28*). These isolates from Vietnam and Hong Kong were associated with a VAG profile of *epf*+/*sly*+/*mrp*+, and the strains from Vietnam were also the cause of meningitis in adults. A unique DNA pattern of pulsotype D, classified into cluster XVI, was found for 3 strains with ST28 isolated from nonfatal cases in this study. Previous studies also reported 1 nonfatal case caused by the ST28 strain from Thailand and Japan (*34*,*38*).

Associations for bacteremic meningitis cases with nonfatal outcomes and probable meningitis cases with fatal outcomes contrasted strikingly in this study. Of 6 fatal cases of probable meningitis, 2 were caused by ST1, 2 by ST25, and 2 by ST104 strains. The extent to which the virulence of each ST strain contributed to these deaths remains uncertain. Another possible explanation may be a frequent involvement of critically ill patients, for whom lumbar puncture was not possible; these patients had probable meningitis and typical meningeal signs, acute disease onset, and positive blood culture only.

Because the clinical charts were retrospectively reviewed and the etiologic diagnosis of *S. suis* infection might not have been readily reported to the attending physicians during the hospitalization of the patients in this study, the extent of investigations of clinical manifestations, possible risk factors, and causes of death might have been limited. Because different physicians were involved in the assessment of different patients in this study, the possibility of misdiagnosis for clinical categories cannot be completely excluded even though meningeal signs and acute disease onset are clinical indicators of meningitis.

In conclusion, this study of the clinical features of 158 cases of *S. suis* seotype 2 infection in humans in Thailand showed that the disease occurs sporadically in adults and results in a mortality rate of ≈9.5%; the major clinical manifestations include meningitis and sepsis. MLST analyses of 165 isolates from humans indicated that the major STs were ST1 followed by ST104. Although both ST1 and ST104 strains cause sepsis, it is likely that only the ST1 strain causes meningitis. Further studies are needed to elucidate the pathogenesis of the human *S. suis* infections that are prevalent in Southeast Asian countries.

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Babesiosis in Lower Hudson Valley, New York, USA

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Although Lyme disease has been endemic to parts of the Lower Hudson Valley of New York, United States, for >2 decades, babesiosis has emerged there only since 2001. The number of Lower Hudson Valley residents in whom babesiosis was diagnosed increased 20-fold, from 6 to 119 cases per year during 2001–2008, compared with an ≈1.6-fold increase for the rest of New York. During 2002–2009, a total of 19 patients with babesiosis were hospitalized on 22 occasions at the regional tertiary care center. Concurrent conditions included advanced age, malignancies, splenectomy, and AIDS. Two patients acquired the infection from blood transfusions and 1 from perinatal exposure, rather than from a tick bite. One patient died. Clinicians should consider babesiosis in persons with fever and hemolytic anemia who have had tick exposure or have received blood products.

Babesiosis is a tick-borne infection of erythrocytes. *Babesia microti*, the most common cause of babesiosis in North America, is transmitted by *Ixodes scapularis* ticks, which also transmits *Borrelia burgdorferi*, the cause of Lyme disease, and *Anaplasma phagocytophilum*, the cause of human granulocytic anaplasmosis (HGA) (*1*,*2*). Babesiosis, however, does not occur in all Lyme disease–endemic areas (*1*). Although Lyme disease has been highly endemic to parts of the Lower Hudson Valley (LHV) of New York in the United States since the mid-1980s, the first indigenous case of babesiosis did not occur there until 2001 (*3*).

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To better characterize the recent emergence of babesiosis in this region, we reviewed data for 2001–2008 on *I. scapularis* tick–transmitted infections in the 7 counties that make up the LHV. These counties are located immediately north of New York City. In addition, we reviewed the medical records of patients with babesiosis who were hospitalized during January 1, 2002–December 31, 2009, at the Westchester Medical Center (WMC), the sole tertiary care medical center in the LHV.

Methods

Reported Babesiosis Cases in the LHV

For this report, we defined the LHV as Westchester, Putnam, Dutchess, Orange, Rockland, Ulster, and Sullivan counties (*4*). Cases of babesiosis, Lyme disease, and HGA in this region were tabulated on the basis of statistics on reportable diseases available on the New York State Department of Health (NYSDOH) website (*5*). Cases listed as ehrlichiosis were assumed to be a surrogate for HGA in this region. For purposes of surveillance by the NYSDOH during the period reviewed, a diagnosis of babesiosis was considered confirmed when 1) a clinically compatible illness occurred in conjunction with identification of *Babesia* spp. parasites on blood smear or a positive immunoglobulin G (or total antibody) *Babesia* spp. serologic titer of ≥256 (with testing confirmed by NYSDOH), or 2) in the absence of a clinically compatible illness, *Babesia* spp. parasites were present on blood smear (*5*).

Patients Hospitalized with Babesiosis at WMC

WMC is located in Valhalla, Westchester County, New York. We retrospectively reviewed medical records of patients with babesiosis documented by peripheral blood smear who were hospitalized at WMC during January 1,

2002–December 31, 2009. Case ascertainment was based on review of microbiology and infectious diseases records. For the 2 patients who had >1 hospitalization for babesiosis, we included data for only the first hospitalization. Complete records were available for all but 1 patient; partial records were available for that patient. The Institutional Review Board at New York Medical College approved the medical records review.

Statistical Methods

Continuous variables were described with means and standard deviations. Categorical variables were described with frequencies and percentages, and differences were compared with the Fisher exact test (2-tailed). Relative risk estimates over time and among counties were computed by using Poisson regression adjusting for population size. A p value <0.05 was considered significant.

Results

The LHV comprises 4 counties west of the Hudson River (Rockland, Orange, Sullivan, and Ulster) and 3 counties east of the Hudson River (Westchester, Putnam, and Dutchess) (Figure 1). Westchester County is located immediately north of the Bronx, New York.

Babesiosis has been a reportable disease in New York since 1986. According to statistics compiled by NYSDOH (*5*), the number of cases of babesiosis diagnosed in residents of the 7-county LHV increased nearly 20-fold from 6 per year to 119 per year during 2001–2008 (Figure 2), with an average increase in incidence of 48.7% per year (95% confidence interval [CI] 40.6%–57.2%) (Table 1) (*5*,*6*). In the rest of the state, the number of cases increased only ≈1.6-fold during the same period (from 89 cases in 2001 to 142 cases in 2008) (*5*).

Although the number of babesiosis cases increased on both sides of the river, 104 (87.4%) of 119 reported cases in 2008 occurred in residents of counties east of the Hudson River (Table 1). The 104 cases in the 3 counties east of the Hudson River, with a total population of 1,346,065 (*6*), corresponds to 7.7 cases per 100,000 residents, compared with 15 cases among a total population of 936,051 or 1.6 cases per 100,000 for the 4 counties west of the Hudson River (relative risk [RR] 4.82, 95% CI 2.79–8.92; p<0.001). In the 3 counties east of the river, Dutchess County accounted for 62 of the babesiosis cases in 2008 (21.2/100,000), Westchester County for 36 cases (3.8/100,000), and Putnam County for 6 cases (6.0/100,000); thus, the prevalence of babesiosis in 2008 was significantly greater for Dutchess County than for Westchester County (RR 5.61, 95% CI 3.72–8.46; p<0.001) or for Dutchess than for Putnam County (RR 3.53, 95% CI 1.51–8.09; p = 0.003). No significant difference was detected between Putnam and Westchester Counties (RR 1.60, 95% CI 0.68–3.81; p = 0.28) (*5*,*6*).

Figure 1. Map of the Lower Hudson Valley of New York, USA. Westchester, Putnam, and Dutchess Counties are east of the Hudson River, and Orange, Rockland, Ulster and Sullivan Counties are west of the Hudson River. The star indicates the site of the Westchester Medical Center. Permission for use of this image granted from the Westchester Institute for Human Development on July 23, 2010.

For purposes of comparison, in 2001, a total of 2,584 Lyme disease cases were reported from the LHV, compared with 4,609 in 2008, representing a <2-fold increase; 78 ehrlichiosis (HGA) cases were reported in 2001, compared with 213 in 2008, a <3-fold increase (*5*). In 2008, 2,369 (51.4%) of the 4,609 reported Lyme disease cases occurred in residents of counties east of the Hudson River, compared with 186 (87.3%) of 213 reported ehrlichiosis (HGA) cases.

Hospitalized Patients with Babesiosis

Coincident with the emergence of babesiosis in the LHV, the number of patients hospitalized at WMC with this infection also markedly increased. Nineteen patients (18 adults) were hospitalized with babesiosis at WMC on 22 occasions from 2002 through 2009. All 19 patients were residents of LHV; 15 (79%) resided in Westchester County, 2 in Dutchess County, and 1 each in Orange and Putnam Counties.

The only child affected was a 6-week-old infant who acquired *B. microti* infection perinatally; a detailed case history for this patient will be reported elsewhere. For 2 of the 18 cases in adults, transfusion of infected blood products

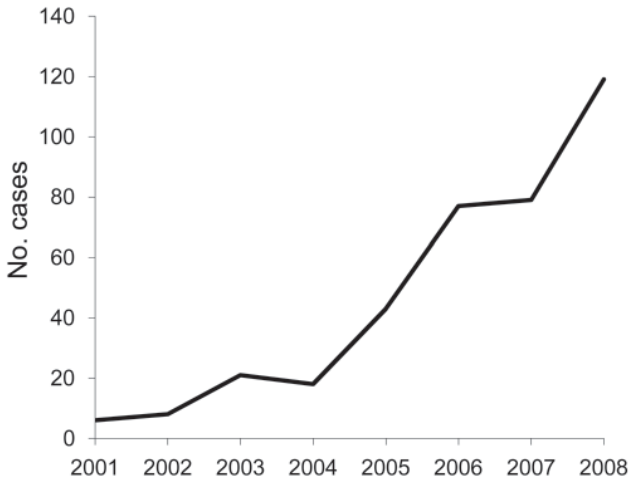

Figure 2. Annual number of reported babesiosis cases, Lower Hudson Valley, New York, USA, 2001–2008.

was believed to have been the route of infection; 1 of these cases is described in more detail elsewhere (*7*). Fifteen (94%) of the 16 other adult patients had potential tick exposure in the LHV (tick exposure is defined as exposure to outdoor environments where ticks are likely to reside); for 10 (67%) of these patients, this was the only known tick exposure within 30 days before onset of symptoms. Of the 16, however, only 3 (19%) actually recalled a tick bite within this 30-day period.

All 18 adult patients had a positive peripheral blood smear for *Babesia* spp. parasites (Table 2). Of the 8 patients who were tested for *B. microti* DNA by PCR, all had positive results. All but 2 of the patients were admitted during May–October. One patient was admitted in December, and the other was admitted in January. The patient who sought care in December had a tick bite 1 month before admission. Thirteen (72%) patients were men; the mean age was 54.1 years (range 21–95 years). Mean time from onset of symptoms to diagnosis was 13.6 days (median 11 days, range 3–33 days).

Five (28%) patients had had a splenectomy before the babesiosis diagnosis, 2 (11%) had AIDS, and 5 (28%) had malignancies (2 of whom were among the 5 patients who had splenectomies). Of the 5 patients with malignancies, 1 had acute myelogenous leukemia and had received a stem cell bone marrow transplant, 2 patients had B-cell follicular lymphoma (and had been treated with rituximab), 1 had a teratoma, and 1 had renal cell carcinoma. Of the 8 patients <50 years of age, 5 (63%) were potentially immunocompromised because of malignancy, splenectomy, or AIDS.

Common symptoms or signs were fever (temperature ≥38°C) (83%), headache (39%), malaise (33%), and chills (28%); splenomegaly was present in 2 (15%) of the 13 patients with an intact spleen. Frequent laboratory findings included anemia, thrombocytopenia, and abnormal liver function tests (Table 2). All 15 patients for whom a lactate dehydrogenase level was available had a value above the upper reference limit (221 U/L). Reticulocytes varied from 1.1% to 19.9% in 12 patients (median 3.1%; reference 0.5%–1.5%). Haptoglobin level was <20 mg/dL in all 10 patients who were tested (reference 26–85 mg/dL).

Eleven patients were treated with azithromycin and atovaquone; a rash to azithromycin developed in 1 patient, and the drug regimen was changed to clindamycin and atovaquone. In another patient, a rash to atovaquone developed, and clindamycin and quinine was prescribed. Six patients were initially treated with clindamycin and quinine; adverse reactions to quinine developed in 3. In 1 patient, QT prolongation developed, and in 2 patients, hearing loss developed. One patient was initially treated with clindamycin and atovaquone. Eight (44%) patients required blood transfusion for anemia, and 3 (17%) received erythrocyte exchange as adjunctive therapy.

Length of hospital stay ranged from 3 to 73 days (median 8 days). One patient had left upper quadrant pain and splenic rupture and was treated conservatively without surgery. The 1 death occurred in a 95-year-old patient in whom shock and respiratory failure developed and who required admission to the intensive care unit. Another patient required ventilator support. In 15 (83%) patients, infection resolved with a single course of antimicrobial drugs. Illness recurred in 2 patients but resolved after a subsequent and more prolonged course of antimicrobial

Table 1. Babesiosis cases reported to the New York State Department of Health, Lower Hudson Valley, New York, USA, 2001–2008

Area (2008 population)	2001	2002	2003	2004	2005	2006	2007	2008
West of Hudson River (936,051)	0	0	1	2	5	7	5	15
Rockland County (298,545)	0	0	0	1	0	2	0	3
Orange County (379,647)	0	0	1	1	1	5	5	7
Sullivan County (76,189)	0	0	0	0	1	0	0	1
Ulster County (181,670)	0	0	0	0	3	0	0	4
East of Hudson River (1,346,065)	6	8	20	16	38	70	74	104
Dutchess County (292,878)	2	4	6	7	23	42	44	62
Putnam County (99,244)	1	0	1	0	2	3	1	6
Westchester County (953,943)	3	4	13	9	13	25	29	36

Table 2. Selected demographic and clinical features and laboratory test results for 18 adults with babesiosis hospitalized at Westchester Medical Center, Valhalla, New York, USA, 2002–2009*

Characteristic	Value
Mean age, y, ± SD (range)	54.1 ± 20.1 (21–95)
Male, no. (%)	13 (72.2)
Mean time from symptom onset to diagnosis, d, ± SD (range)	13.6 ± 9.28 (3–33)
Recollection of tick bite within 30 d, no. (%), n = 16	3 (18.8)
Temperature >38°C, no. (%)	15 (83.3)
Splenomegaly, no. (%), n = 13	2 (15.4)
Mean initial parasitemia, %, ± SD (range), n = 17†	4.49 ± 4.57 (0.01–14)
Mean highest level of parasitemia, %, ± SD (range), n = 17†	5.34 ± 5.79 (0.05–18)
Mean initial leukocyte count × 10^9/L,± SD (range), n = 17	7.2 ± 3.38 (3.2–15.4)
Lymphocyte count <1,000 ×10^6/L, no. (%), n = 12	5 (41.6)
Mean hemoglobin minimum, g/dL,± SD (range)	8.2 ± 1.98 (3.5–11.1)
Mean platelets minimum, × 10^9/L, ± SD (range)	110.8 ± 139.2 (19–615)
Platelets minimum <150 × 10^9/L, no. (%)	16 (88.9)
Mean initial erythrocyte sedimentation rate, mm/h,± SD (range), n = 9	76.7 ± 33.3 (32–138)
Mean initial lactate dehydrogenase, U/L, ± SD (range), n = 15	931.5 ± 562 (229–2074)
Mean initial aspartate aminotransferase, U/L, ± SD (range)	237.7 ± 366.9 (19–1450)
Initial aspartate aminotransferase >30 U/L, no. (%)	14 (77.8)
Mean initial alanine aminotransferase, U/L, ± SD (range)	110.2 ± 111 (16–433)
Initial alanine aminotransferase >40 U/L, no. (%)	13 (72.2)
Mean initial total bilirubin, mg/dL, ± SD (range)	3.4 ± 5.59 (0.4–24.6)
Initial total bilirubin >1.2 mg/dL, no. (%)	10 (55.6)
Mean serum sodium minimum, meq/L, ± SD (range), n = 17	127.6 ± 10.1 (94–139)
Mean creatinine maximum , ng/mL, ± SD (range), n = 17	1.3 ± 0.59 (0.7–2.7)

*Data were obtained from all 18 patients unless otherwise indicated.
†For 1 patient with a positive smear, the level of parasitemia is unknown.

drug treatment (the 2 latter patients have been included in previous reports [*7*–*9*]).

Discussion

As of 2008, babesiosis cases in residents of the LHV accounted for 45.6% of the 261 cases reported in New York (*5*). Testing of selected *I. scapularis* ticks by PCR in 2002 showed positive results for *B. microti* in tick pools collected in Dutchess and Westchester Counties (*5*). A more recent study of 154 adult *I. scapularis* ticks collected in 2008 from 2 locations in Westchester County identified 24 (15.6%) ticks that were infected with *B. microti* according to PCR, compared with 34 (25.8%) of 132 adult ticks collected from 3 locations in Suffolk County, in Long Island, New York ($p<0.04$) (*10*); babesiosis has been indigenous to Suffolk County since 1975, with 95 cases reported there in 2008 alone (*5*). These infection rates, however, should be interpreted cautiously because an unknown proportion of positive findings may have resulted from detection of *B. odocoilei* in the ticks evaluated, rather than *B. microti*. *B. odocoilei*, which is not regarded as a human pathogen, infects deer ticks more frequently than does *B. microti* in sites where these piroplasms coexist (*11*).

There are 2 prior reports of hospitalized patients in New York with babesiosis. One report published in 1998 described 139 adults with babesiosis hospitalized during 1982–1993 (*12*). More than 90% of these patients resided in Suffolk County; only 2 resided in Westchester County. The other report, published in 2001, described 34 adults and children with babesiosis hospitalized at 2 tertiary care centers in Suffolk County (*13*). The latter patients were hospitalized over 13 consecutive years, but the exact years were not specified. The general clinical and laboratory features of babesiosis in these 2 case series were similar to those observed in the patients in our study. Most patients had a nonspecific febrile illness associated with hemolytic anemia, thrombocytopenia, and abnormal liver function test results. Of the 139 patients in the 1998 series, 16 (11.7%) had had a splenectomy (*12*), as did 8 (27%) of the 30 adults in the 2001 report (*13*), but in neither of the 2 earlier reports were any patients identified as having lymphoma and receiving treatment with rituximab (*9*), receiving a transplantation, or having AIDS. Thus, our case series presumably included more patients now recognized to be at high risk for relapse of infection (*9*). The 5.6% case-fatality rate in our study, however, is slightly lower than the 6.5% in the 1998 report (*12*) and the 8.8% in the 2001 report (*13*). Unlike the 2 prior case series, 2 (11%) of the patients in our study were believed to have been infected through receipt of an infected blood product (*7*), which provides further evidence of the growing importance of this route of transmission (*14*–*18*).

Six (33%) of the patients reported here had serologic evidence of Lyme disease, but this finding may overestimate the frequency of coinfection because only 1 had an objective clinical manifestation of Lyme disease (erythema migrans).

Among the adult ticks collected in Westchester County in 2008 (*10*), 79.2% of those infected with *Babesia* spp. were also infected with *B. burgdorferi*, which reinforces the need to consider the possibility of concomitant Lyme disease in patients from the LHV in whom babesiosis is diagnosed.

How *B. microti* found its way from areas to which this microorganism is endemic into the *I. scapularis* tick population of the LHV is unclear. Evidence suggests that babesiosis is also emerging as a human pathogen in contiguous geographic areas of western Connecticut (*19*,*20*). The principal animal reservoir for *B. microti* is the white-footed mouse, *Peromyscus leucopus* (*1*). Other reservoirs include voles and shrews. These animals are not likely to travel great distances, which suggests that movement of these animals is an unlikely explanation for the emergence of babesiosis in the LHV.

Babesiosis is an emerging infectious disease in the LHV of New York with the potential to cause serious illness and death, especially in highly immunocompromised patients. Clinicians should consider this diagnosis in persons with fever and hemolytic anemia who have been exposed to ticks or have received blood products.

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Experimental Oral Transmission of Atypical Scrapie to Sheep

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To investigate the possibility of oral transmission of atypical scrapie in sheep and determine the distribution of infectivity in the animals' peripheral tissues, we challenged neonatal lambs orally with atypical scrapie; they were then killed at 12 or 24 months. Screening test results were negative for disease-specific prion protein in all but 2 recipients; they had positive results for examination of brain, but negative for peripheral tissues. Infectivity of brain, distal ileum, and spleen from all animals was assessed in mouse bioassays; positive results were obtained from tissues that had negative results on screening. These findings demonstrate that atypical scrapie can be transmitted orally and indicate that it has the potential for natural transmission and iatrogenic spread through animal feed. Detection of infectivity in tissues negative by current surveillance methods indicates that diagnostic sensitivity is suboptimal for atypical scrapie, and potentially infectious material may be able to pass into the human food chain.

Since the discovery of atypical scrapie (*1*) and its subsequent identification, mostly through active surveillance, in several countries (some with no previous history of transmissible spongiform encephalopathies [TSEs]) (*2*,*3*) such as New Zealand (*4*) and Australia (*5*), scientists have debated whether this form of TSE is in fact spontaneous or acquired (*3*,*4*,*6*–*8*) rather than contagious. The epidemiologic studies that have been undertaken suggest that atypical scrapie does not appear be transmitted between animals in the field situation (*7*,*8*). Although the routes by which natural transmission occurs have never been fully established for TSEs, it is widely accepted that ingestion of infective material, i.e., the oral route, is a key component in some TSEs, e.g., kuru (*9*), variant Creutzfeldt-Jakob disease (*10*), bovine spongiform encephalopathy (*11*), and transmissible mink encephalopathy (*12*).

Within the sheep population, susceptibility to particular strains of TSE has been shown to be heavily affected by polymorphisms of the prion protein gene of the sheep (*13*–*18*). The successful transmission of atypical scrapie to sheep after intracerebral inoculation has been previously reported for sheep of 1 genotype ($A_{136}H_{154}Q_{171}/A_{136}H_{154}Q_{171}$) (*19*,*20*), and challenges in other homologous and heterologous genotype combinations are ongoing. However, successful intracerebral transmission of a particular TSE agent in a particular species does not necessarily indicate susceptibility by the oral route (*21*).

The tissue distribution of infectivity or disease-specific prion protein (PrP^{Sc}) in bovine spongiform encephalopathy in sheep has led to extensive public health control measures based on the known pathogenesis and distribution of PrP^{Sc} in edible tissues, and their removal from carcasses of animals over a certain age. Classic scrapie may also show the widespread accumulation of PrP^{Sc} in peripheral tissues. Although early studies of atypical scrapie did not show PrP^{Sc} or infectivity outside the brain, recent data indicate that peripheral tissues from naturally infected animals can harbor infectivity either in the presence or absence of PrP^{Sc} (*22*). However, whether this infectivity is established before or after the agent has propagated in the central nervous system is unknown.

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The first aim of the current study was to examine the distribution of infectivity in peripheral tissues in animals at and beyond the cutoff point for the current meat hygiene regulations of the European Commission (i.e., 12 months of age). The second aim was to investigate the potential for oral transmission of atypical scrapie.

Methods

Recipient Animals

Six Cheviot lambs with PrP genotype AHQ/AHQ and 6 Poll Dorset lambs with the genotype ARR/ARR were sourced from the New Zealand–derived flock owned by the UK Department of Food, Environment and Rural Affairs (*3*), and transported in utero with their dams to the Veterinary Laboratories Agency–Weybridge before lambing. All procedures involving animals were carried out in accordance with the UK Animal (Scientific Procedures) Act 1986, under license from the UK Government Home Office.

Experimental Challenge

Within 24 hours of birth, during February–March 2008, each lamb was challenged orally with 2.5 g of brain homogenate prepared from animals with naturally acquired cases of atypical scrapie of the same genotypes, and again with a further 2.5 g of the same homogenate 14 days later. Homogenate was delivered through a syringe to the oropharynx (*23*). The lambs were kept with their dams and separate from each other until they were weaned at 9–11 weeks of age; after weaning, they were housed in groups according to genotype.

Donor Animals

Inoculum ARRa

This animal was identified through passive surveillance in 2004. The sample was submitted before ELISA screening was used routinely for passive surveillance, and no inoculum remained for further biochemical characterization after the challenge of 5 lambs. Results of immunolabeling in the medulla, cerebellum, and thalamus, and Western blot on the medulla were consistent with atypical scrapie (*24*) (data not shown).

Inoculum ARRb

This animal was identified through active surveillance (fallen stock). ELISA (Bio-Rad Laboratories, Marnes-la-Coquette, France) of this inoculum gave optical density (OD) values of 1.554 and 0.761. The OD of the initial diagnostic screening sample was 1.074. Results of Western blot and immunolabeling throughout the neuraxis were consistent with atypical scrapie (data not shown).

Inoculum AHQ

This animal was identified through active surveillance (fallen stock). ELISA (Bio-Rad Laboratories) of inoculum prepared from this animal gave a positive OD reading of 2.272. The initial diagnostic screening sample gave an ELISA OD of 0.948. Western blot and immunolabelling results were consistent with atypical scrapie, but with a preponderance of white matter staining and substantially less neuropil staining than has been seen in other AHQ atypical cases (data not shown).

Clinical Monitoring

All animals were monitored daily during routine husbandry procedures and monthly during blood sampling from 8 months post inoculation. Within 12 days of the cull date for each sheep, a clinical and neurologic examination was carried out, including cranial nerve assessment and testing of response to scratching of the back according to published protocols (*23*). On the basis of clinical signs, the animal's clinical TSE status was categorized as follows: normal (no apparent signs of scrapie), inconclusive with regards to scrapie (e.g., impaired menace response, minor wool loss), and suspected scrapie (combination of abnormalities in behavior, sensation, or movement), as has been described for goats (*25*).

Cull Schedule and Sampling

Three animals of each genotype were selected at random and culled at 12 months of age, and the remaining 3 were culled at 24 months of age. Animals were euthanized by using quinalbarbitone sodium (Somulose–Dechra Veterinary Products, Shrewsbury, UK), and a variety of samples of neural and non-neural tissues (online Appendix Table, www.cdc.gov/EID/content/17/5/848-appT.htm) were obtained by using aseptic techniques and either placed into 10% formal saline (neural tissues), 10% buffered formalin (non-neural tissues) or frozen and held at –80°C for subsequent examination by immunohistochemical (IHC) testing, ELISA, or both (Table), depending on the size and nature of the sample. Some samples were collected for examination in the event of a positive result in the samples chosen for initial screening.

Immunohistochemical Testing

Fixed samples were processed into paraffin wax, sectioned, and stained with hematoxylin and eosin as described (*26*). IHC labeling to detect PrP^{Sc} was performed as previously described (*19*) by using mouse monoclonal antibody 2G11 (Institut Pourquier, Montpellier, France), raised against ovine PrP peptide sequence 146-R154 R171–182. IHC profiles were created by using a standard subjective method as previously described (*24*) in which the type of PrP^{Sc} immunolabeling is assessed in a standard

range of precise neuro-anatomical areas. Tissues from the lympho-reticular system of each challenged animal were examined by IHC as described above.

ELISA

All tissues were analyzed by using the TeSeE kit (Bio-Rad Laboratories) for TSE detection in sheep and goats, according to the manufacturer's instructions. The cutoff value was calculated as the average absorbance reading of the negative control values plus a value of 0.14 absorbance units. To directly measure PrP^{Sc} content in the samples that were used to inoculate the recipient animals, the inocula were prepared for the Bio-Rad protocol before analysis to account for variation in tissue and buffer content. Samples were centrifuged at high speed to concentrate insoluble material. The weight of the pellet was calculated, and then it was resuspended in homogenization buffer to prepare a 20% wt/vol homogenate and continued as described from the ribolysation stage. Pellets that contained insufficient weight to prepare 250 μL of 20% wt/vol homogenate were supplemented by using brain homogenate or relevant tissue prepared from sheep that had not been exposed to scrapie (control reference material) before testing.

Western Blot

Positive, fresh brain samples were subjected to the TeSeE Universal Western blot (Bio-Rad Laboratories, catalog no. 355 1169) as described (*19*). Molecular mass markers were included at either end of the gel. A single lane each of samples from animals with known UK classic scrapie, known UK bovine BSE, and a known UK atypical scrapie were included for profile comparisons.

Mouse Bioassay

Brain areas from animals positive for PrP^{Sc} were assayed in mice to assess the stability of agent following passage. In addition, bioassay of 3 additional tissues (cerebellum, spleen, and distal ileum) has also been initiated from every challenged sheep whether all tests were negative or not (online Appendix Table). Samples from the donor and experimental recipient sheep were treated in the same way. Tissue homogenate (10%) was prepared wt/vol in normal saline, screened for microbiologic sterility by using standard methods, treated using ampicillin and gentamicin if contamination was identified, and rechecked before use.

Panels of 10 transgenic mice overexpressing ovine prion protein gene (Tg338 [*27*]) were inoculated intracerebrally with 20 μL and, when sufficient inoculum was available, intraperitoneally with 100 μL of homogenate. Mice were monitored weekly and were killed when they had shown clinical signs on 2 of 3 consecutive monitoring days or had reached their natural lifespan. Brains were then fixed and processed, and lesion profiles were produced as described in detail elsewhere (*28*).

Results

Full details of genotype, clinical status, kill time points, and test results for each animal are shown in the online Appendix Table. A proportion of the mouse infectivity bioassays are incomplete, as indicated in the text below, so some tissues that are currently considered negative for PrP^{Sc} by bioassay may change status at a later date.

Cull at 12 Months

Of the 6 animals (3 ARR/ARR and 3 AHQ/AHQ) killed after 12 months, 5 were clinically healthy at the time of culling. Animal 3 appeared nervous during handling, although its behavior at previous blood sampling sessions had been unremarkable, and it displayed a bilaterally absent menace response (inconclusive results with regards to scrapie).

None of the sheep exhibited PrP^{Sc} in the tissues examined. Bioassays in mice of the cerebellum, spleen, and distal ileum from each animal are ongoing. To date, infectivity has been demonstrated in the cerebellum of animal 1 and the distal ileum of animals 8 and 9. Only the bioassay from animal 9 has been completed, with 9 mice succumbing to disease (Figure 1). A proportion of the mice used in the assays of samples from animals 1 and 8 are still alive.

Cull at 24 Months

Of the 6 sheep culled at 24 months post inoculation, 3 appeared clinically normal. Animal 6 displayed alopecia suggestive of pruritus although no pruritic behavior was observed (inconclusive with regards to scrapie). Animal 10 appeared nervous when it was approached and handled, which had not been observed on previous blood sampling sessions, and displayed a wide-based hind limb posture (inconclusive with regards to scrapie). Animal 12 appeared nervous with a fine head tremor during handling, had a wide-based hind limb posture, and was ataxic with uncoordinated jumps, swaying, and loss of balance (online Appendix Video, www.cdc.gov/EID/content/17/5/848-appV.htm). At blood sampling 2 months earlier, the sheep had been observed circling clockwise when left alone, which was not seen at the final examination (suspected scrapie). None of the sheep had a positive scratch test, and none of these 6 sheep had lost weight before cull.

PrP^{Sc} was detected in 2 animals only, both of which were AHQ homozygotes. In animal 11, which was clinically normal, PrP^{Sc} was observed in the caudal thalamus, restricted to the lamina medullaris externa, in the caudate nucleus, the amygdala, and external capsule, and minimal labeling was found in the basal and septal nuclei. Positive

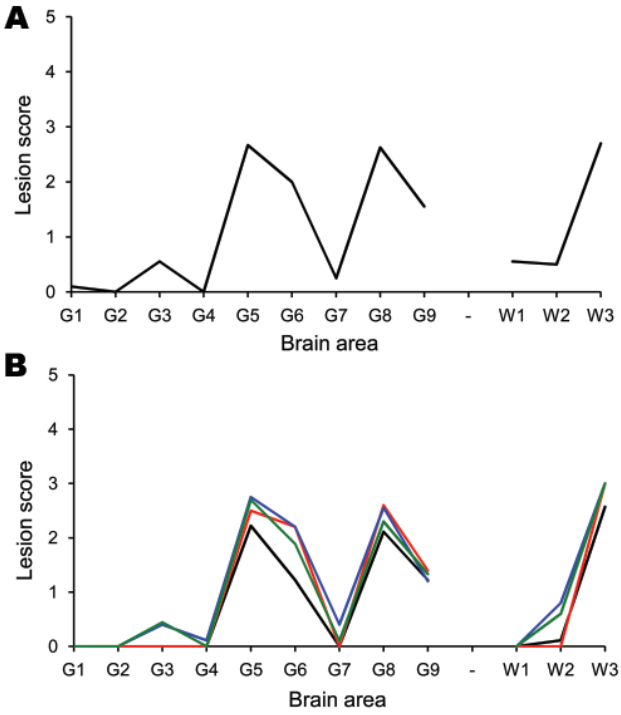

Figure 1. Tg338 mouse vacuolation lesion profiles of mice infected with scrapie. Only clinically affected mice were considered when generating the lesion profiles. Number in parentheses indicates the mean incubation period of the mice, which contributed to the lesion profile. A) The donor AHQ/AHQ sheep (177 ± 3; n = 10). This profile is compatible with that obtained from other naturally-occurring cases of atypical scrapie (*19*,*20*). B) Recipient sheep brain and distal ileum. Cerebellum from animal 12 (179 ± 12; n = 6/10) is indicated in red. Basal ganglion from animal 11 (193 ± 7; n = 10/10) is indicated in blue. Hippocampus from animal 11 (198 ± 11; n = 10/10) is indicated in green. Distal ileum from animal 9 (247 ± 23; n = 9/9) is indicated in black.

immunolabeling (not shown) was seen in the hippocampus, together with extensive incidental "thready" staining (*29*). White matter labeling was found in the olfactory tract and rostral commissure (Figure 2). No PrP^{Sc} was detected in the medulla or cerebellum, the areas currently used for statutory surveillance purposes. In animal 12, PrP^{Sc} was distributed widely throughout the brain and was detectable by IHC (Figure 2), ELISA, and Western blot (Figure 3). Widespread white matter labeling and mild-to-moderate granular labeling were found, consistent with that described for natural cases of atypical scrapie (*24*) (Figure 2). No evidence of PrP^{Sc} accumulation was found in any of the examined tissues from the other animals. Western blot profiles of PrP^{Sc} from these animals were compatible with those of animals with of atypical scrapie (Figure 3).

In addition to the tissues listed in the Table, the jejunum, lateral retropharyngeal lymph node, respiratory epithelium, triceps muscle, cranial cervical ganglion, nodose ganglion, facial nerve, trigeminal ganglion, and sciatic nerve have all been screened by IHC in the PrP^{Sc}-positive animals. No evidence of PrP^{Sc} accumulation outside the brain has been identified in these animals.

Bioassays of cerebellum, spleen, distal ileum, and of tissues with detectable PrP^{Sc} are still ongoing. To date, the cerebellum sample from animal 11 and the distal ileum samples from animal 12 have resulted in PrP^{Sc}-positive mice, although no PrP^{Sc} was detected in those tissues (online Appendix Table). When assays are complete, the lesion profiles obtained in the mice show that the biological profile of the experimental animals is the same as that of the donor, and that infectivity detected in the peripheral tissues has the same biologic signature as that in the brain (Figure 1).

Discussion

This study is still ongoing and will not be completed until 2012. However, the current interim report documents the successful oral transmission of atypical scrapie, confirms that the disease phenotype is retained following transmission by this route in AHQ/AHQ sheep, and indicates that infectivity can be demonstrated in the gut in the absence of detectable PrP^{Sc} at least as early as 12 months after exposure.

One sheep (animal 12) culled at 24 months post inoculation displayed abnormalities in behavior and movement suggestive of atypical scrapie. Signs like ataxia with head tremor and circling have been described in experimental (*19*) and natural (*3*,*30*) disease, which was attributed to lesions in the cerebellum and forebrain, respectively, corresponding with PrP^{Sc} accumulation in these areas (*20*,*24*).

By contrast, animal 11, which had confirmed atypical scrapie based on postmortem tests, was considered clinically normal. The less severe and limited PrP^{Sc} accumulation in the brain of this sheep than in animal 12 may explain the absence of clinical abnormalities, which is supported by our findings in goats with scrapie in which more extensive PrP^{Sc} accumulation in the brain was usually associated with a more severe clinical disease (*25*).

Although all TSEs are transmissible after intracerebral challenge to a susceptible host, only some are infectious under natural conditions. Therefore, it was important from a pathogenesis and disease control perspective to establish whether or not oral transmission can be successful. However, the challenge model in this study exposed animals as neonates, when the esophageal groove is operational and the lambs are physiologically monogastric. Exposure of 3-month-old ruminating animals to similar amounts of positive brain by the oral route have so far not resulted in any clinical disease, with all animals still alive >1,500 days post challenge (M.M. Simmons, unpub. data), but most

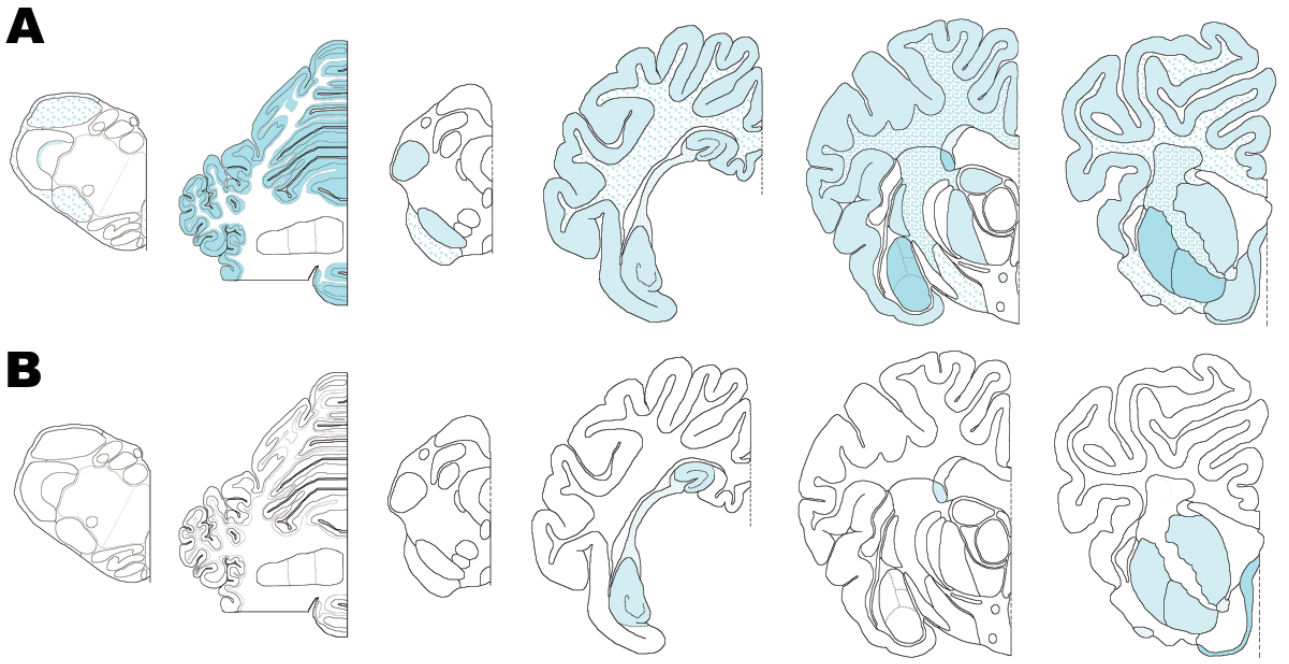

Figure 2. Distribution of immunolabeling in sheep infected with scrapie. A) animal 12, B) animal 11. Animal 12 exhibits the same distribution and type of immunolabeling as seen in natural disease (*23*). In animal 11, immunolabelling was much more restricted and did not involve the cerebellum.

natural cases have been recorded in animals older than this, so these animals may still progress to disease in the next few years. Since this challenge study in older animals has no time-kill component, and no losses caused by unrelated disease have occurred, whether any of these sheep are in a preclinical phase of disease is unknown. Unfortunately, the absence of detectable PrP^{Sc} in lymphoreticular tissues of sheep with atypical scrapie precludes the use of biopsies to ascertain early infection in these animals.

Transmission may be more efficient in newborn animals; the incubation periods of sheep orally infected with classical scrapie were significantly shorter in sheep challenged at 14 days of age than those challenged at 6 months of age (*31*). If, however, oral transmission is only effective in such young animals, then field exposure would most likely have to be through milk, which is known to be a highly effective route of transmission for classical scrapie (*32*). No data are currently available on the potential infectivity of milk from animals with atypical scrapie.

Successful oral transmission also raises questions regarding the pathogenesis of this form of disease. There must be passage of the infectious agent from the alimentary canal to the brain through one of several possible routes, most likely those that have been suggested and discussed in detail for other TSEs, for example, retrograde neuronal transportation either directly (*33*–*35*) or through lymphoid structures or hematogenously (*36*). Infectivity in the absence of readily demonstrable PrP^{Sc} has been reported (*37*–*39*), and although the mouse bioassay may detect evidence of disease in other tissues, these data may not be available for at least another 2 years. More protease-sensitive forms of PrP^{Sc} may be broken down more efficiently within cells and thus do not accumulate in peripheral tissues (*19*), enabling atypical PrP^{Sc} to transit the digestive tract and disseminate through other systems in small amounts before accumulating detectably in the central nervous system.

Although we do not have epidemiologic evidence that supports the efficient spread of disease in the field, these data imply that disease is potentially transmissible under field situations and that spread through animal feed may be possible if the current feed restrictions were to be relaxed. Additionally, almost no data are available on the potential for atypical scrapie to transmit to other food animal species, certainly by the oral route. However, work with transgenic mice has demonstrated the potential susceptibility of pigs, with the disturbing finding that the biochemical properties of the resulting PrP^{Sc} have changed on transmission (*40*). The implications of this observation for subsequent transmission and host target range are currently unknown.

How reassuring is this absence of detectable PrP^{Sc} from a public health perspective? The bioassays performed in this study are not titrations, so the infectious load of the positive gut tissues cannot be quantified, although infectivity has been shown unequivocally. No experimental

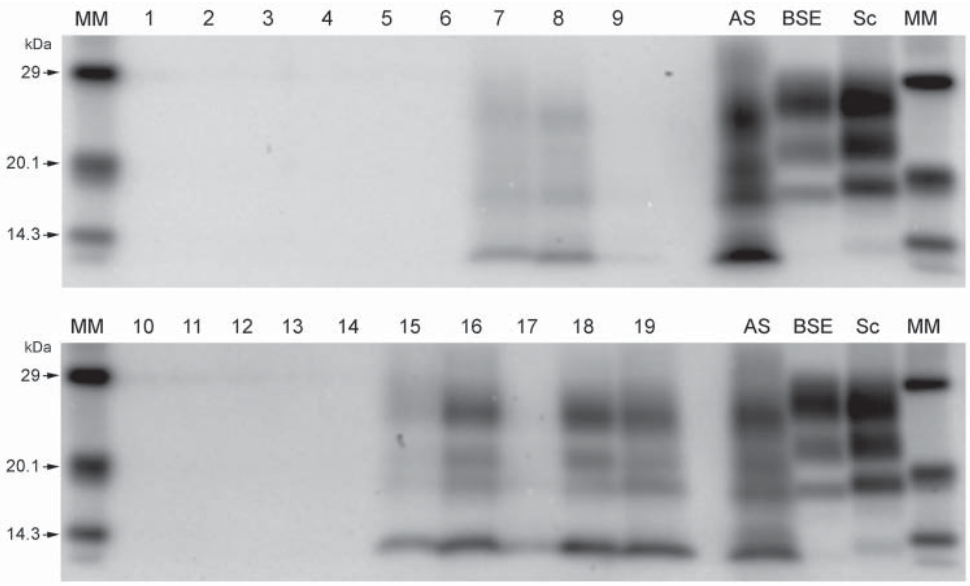

Figure 3. Western immunoblots showing clear atypical scrapie profiles in sheep in the following brain regions; brainstem of donor ARRa (lane 7), frontal cortex of donor ARRb (lane 8) and frontal cortex of donor AHQ (lane 19). The hippocampus and basal nuclei of recipient animal 11 (lanes 15 and 16, respectively) and cerebellum of recipient animal 12 (lane 18). No discernible signal was seen in the medulla of donor ARRb (lane 9), and only a faint profile was visible for the obex of recipient animal 12 (lane 17). Lane 1, animal 2 obex; lane 2, animal 1 obex; lane 3, animal 3 obex; lane 4, animal 4 obex; lane 5, animal 5 obex; lane 6, animal 6 obex ; lane 7, donor ARRa rostral B.stem; lane 8, donor ARRb frontal cortex; lane 9, donor ARRb caudal medulla; lane 10, animal 7 obex; lane 11, animal 8 obex; lane 12, animal 9 obex; lane 13, animal 10 obex; lane 14, case 11 obex; lane 15, animal 11 hippocampus; lane 1, animal 11 basal nuclei; lane 17, animal 12 obex; lane 18, animal 12 cerebellum; lane 19, donor AHQ frontal cortex; AS, atypical scrapie; BSE, classical bovine spongiform encephalopathy; Sc, classical scrapie; MM, molecular mass marker.

data are currently available on the zoonotic potential of atypical scrapie, either through experimental challenge of humanized mice or any meaningful epidemiologic correlation with human forms of TSE. However, the detection of infectivity in the distal ileum of animals as young as 12 months, in which all the tissues tested were negative for PrP^{Sc} by the currently available screening and confirmatory diagnostic tests, indicates that the diagnostic sensitivity of current surveillance methods is suboptimal for detecting atypical scrapie and that potentially infectious material may be able to pass into the human food chain undetected.

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Evidence of Tungiasis in Pre-Hispanic America

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Ancient parasites of the genus *Tunga* originated in America and, during the first half of the 19th century, were transported to the Eastern Hemisphere on transatlantic voyages. Although they were first documented by Spanish chroniclers after the arrival of Columbus, little is known about their presence in pre-Hispanic America. To evaluate the antiquity of tungiasis in America, we assessed several kinds of early documentation, including written evidence and pre-Incan earthenware reproductions. We identified 17 written documents and 4 anthropomorphic figures, of which 3 originated from the Chimu culture and 1 from the Maranga culture. Tungiasis has been endemic to Peru for at least 14 centuries. We also identified a pottery fragment during this study. This fragment is the fourth representation of tungiasis in pre-Hispanic America identified and provides explicit evidence of disease endemicity in ancient Peru.

Tungiasis, a parasitic skin disease that occurs in tropical countries, is caused by sand fleas of the genus *Tunga* (Insecta, Siphonaptera, Tungidae). The disease has recently attracted attention because of high rates of infection for impoverished communities in South America and sub-Saharan Africa and because of new cases reported worldwide as exotic infections among travelers returning to North America and Europe from disease-endemic areas (*1*,*2*). The first species, *Tunga penetrans*, was described by Linnaeus in the 18th century (*Pulex penetrans*, Linnaeus 1758) (*3*), and a second *Tunga* species, which infects humans (*T. trimamillata*), was taxonomically described by Pampiglione et al. in 2002 (*4*,*5*). Studies describing the entomology and pathogenesis of both species are now abundant (*6*,*7*). However, studies describing the recognition, proper identification, and distribution of the species in Ecuador and Peru, the only 2 countries where the second new species has been reported, are lacking (*8*).

Before the Spanish Conquest, nuclear America was a geopolitical area where the main indigenous populations and cultures were located and where cultural development took place more rapidly than anywhere else in the Americas. It was composed of the 2 centers of the New World, Mesoamerica (Aztecs and Mayas) in the north and the Andean Area (Incas) in the south. The Inca Empire (AD 1430–1532), or Tahuantinsuyo, occupied an extensive territory of South America; at the time of the Spanish Conquest by Francisco Pizarro's troops in 1532, the Inca Empire stretched from southern Colombia to northern Argentina and central Chile and included the countries now known as Ecuador, Peru, and Bolivia (Figure 1, panel A). The Tahuantinsuyo was the most organized civilization in pre-Hispanic America and was characterized by substantial technologic advances in agriculture, architecture, and pottery, which were inherited from their ancestors and conquered tribes (*9*). Most pre-Hispanic anthropologic evidence originates from Inca predecessors, ancient cultures of Peru that were technologically advanced and developed pottery many centuries before the collapse of the Incan civilization (*10*).

Although tungiasis was recognized and documented by Spanish chroniclers shortly after the arrival of Columbus in Central America in 1492 (*11*), the South American ancestors of the Incas distinguished this affliction from others and depicted it on clay jars, pottery, and ceramics, called *huacos* in Peru (*12*–*14*). Many other autochthonous diseases of ancient Peruvians have also been portrayed on anthropomorphic vessels, thus providing indirect evidence of their presence in this part of the continent (*15*). Most

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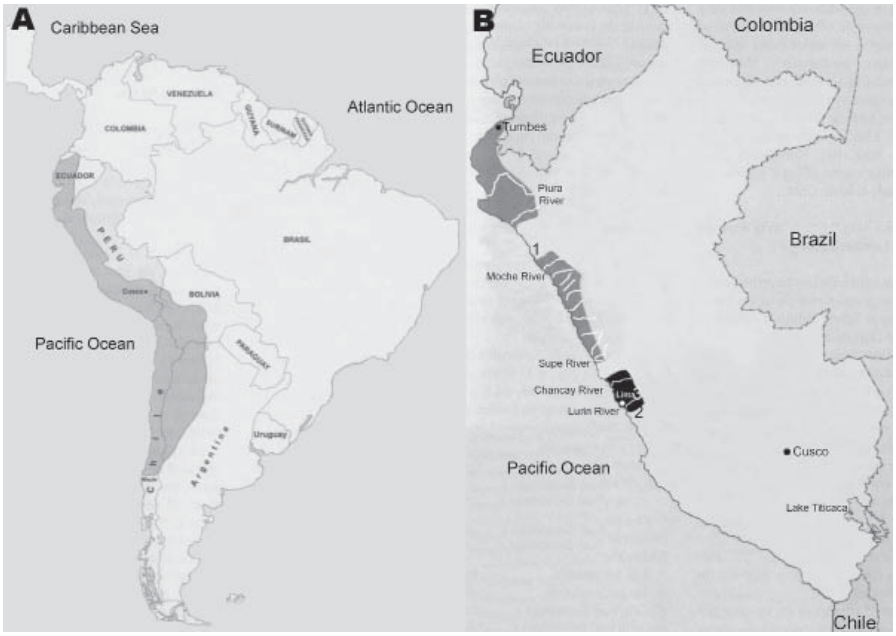

Figure 1. A) Geopolitical map of the Incan Empire at the time of its greatest expansion (dark gray shading). B) Geographic location of the Chimu (dark gray shading) and Maranga (black shading) cultures in modern Peru. The numbers indicate the sites at which pre-Incan anthropomorphic potteries depicting tungiasis were located: 1, Chicama Valley; 2, Pachacamac Valley; 3, Surquillo.

of this pottery was initially discarded by the Spanish invaders, who looted sacred places, temples, and tombs in their search for gold in the mid-16th century. However, at the turn of the century, interest in pre-Incan cultures and their legacy increased, and these anthropologic pieces represented a cornerstone for understanding the dynamics of cultures that antedated the Incas.

Our objective was to evaluate the antiquity of tungiasis in pre-Hispanic America through the assessment of different kinds of early documentation from 1 of the most advanced civilizations of pre-Hispanic America, which was in Peru. Because documentation of the tungiasis presence in Peru is scarce, we conducted an extensive retrospective search that involved the critical appraisal and inspection of 2 main classes of materials: written evidence and earthenware representations.

During our studies, a pottery fragment was newly identified in a collection storage facility at the Amano Museum Foundation in Lima, Peru. This unique polychromic fragment is the fourth earthenware representation of early tungiasis in Peru identified to date and the only one that represents the different stages of *Tunga* spp. infection, which distinguishes it from previously described pre-Incan pottery.

The Search

To critically inspect written evidence and cover all available information relating to the presence of *Tunga* spp. in Peru, we searched for all documented names ascribed to this parasite over the past 4 centuries (*16*–*19*). We used 35 local terms (*nigua, nihua, niua, pique, pigue, piqui, piki, pico, sico, seccec, chegoe, chego, chigger, puce-chique, puce de sable, chique, chica, bicho de pé, bicho do porco, pulga de areia, jatecuba, jigger, chicque, sand flea, tchike, tschike, sike, xique, ckicke, aagrani, atten, tom, tü, tungay,* and *tunga*) and 9 scientific terms (*Pulex minimus cutem penetrans Americanus*, *Pulex minutissimun nigricans*, *Acarus fuscus sub cutem nidulans proboscide acutiore*, *Pulex penetrans*, *Rhynchoprion penetrans*, *Sarcophaga penetrans*, *Dermatophilus penetrans*, *Sarcopsylla penetrans*, and *Tunga penetrans*). Using on-site electronic catalogs, we screened all available manuscripts, books, doctoral theses, journals, bulletins, monographs, and periodicals in their original English, Spanish, or French from 2 major sources: the Main Campus Library of the School of Medicine at Cayetano Heredia Peruvian University, in Lima, Peru, and the William H. Welch Medical Library, Institute of History of Medicine at Johns Hopkins University, in Baltimore, Maryland, USA. These searches were complemented by using the PubMed, LILACS, Scielo, and Medline electronic databases with no publication date- or language-based restrictions. Digitized and printed materials were screened.

After screening the written material to identify the locations of ceramics portraying tungiasis, we assessed earthenware representations through visits to selected private collections of pre-Incan pottery at the Amano Museum Foundation in Miraflores, Lima, Peru, and the Halls of Mexico, Central and South American Peoples at the American Museum of Natural History in New York, New York, USA. These museums were the only facilities cited at least 1 time as potential depositories of pottery depicting pre-Incan tungiasis. All anthropomorphic ceramics that depicted ≥1 nodule-like representations on the lower or

upper extremities, either localized or clustered, with or without representations of holes in the soles of the feet and irrespective of the presence of a central depression, were deemed possible depictions of *Tunga* spp. infection. From each museum, ≈50 pieces were screened; data on the date and location of findings were recorded when they fulfilled the criteria for possible depiction of tungiasis. A complete screening of the entire collection of ceramics representing diseases of Ancient Peruvians was possible only at the Amano Museum Foundation.

Search Results

We found written evidence of tungiasis in pre-Incan or Incan times in 17 documents (7 in English, 4 in French, and 6 in Spanish) (Table 1). The documents were 1 unique 17th-century manuscript written by the indigenous Peruvian chronicler Guaman Poma de Ayala (finalized during 1615–1616), 1 monograph, 1 bulletin, 2 doctoral theses, 5 books, and 7 journals. The timeframe in which these documents were written extends from 1615 through 1990.

As for the earthenware representations, we identified 4 anthropomorphic figures representing pre-Incan tungiasis (Table 2). Of these 4 figures, 3 were reproduced in the written materials surveyed (1 from an unknown location and 2 from the American Museum of Natural History), and 1 was a piece of polychromic ceramic, located in the Amano Museum Foundation, which had not been previously described.

The anthropomorphic pottery shown in Figure 2 originated from the Chimu Culture (c. AD 1200–1470). It is a single-spout bottle that represents a man holding a pointed object and depicts multiple holes in the sole of

Table 1. Sources of written evidence of tungiasis in pre-Incan times*

Reference	Type of publication	Original language	Term for *Tunga* spp.	Chapter or article name and pages
Guaman Poma de Ayala, 1615/1616	Manuscript†	Spanish	*Pique niua*	Primer Capítvlo d los Ingas: Armas Propias. Milagro de Dios: p. 95
(*16*)	Thesis	French	*Chique, nigua, seccec*	Chapitre II. Du Pulex penetrans, Chique ou Nigua: pp. 62–113
(*17*)	Monograph	French	*Chique, seccec*	Introduction: p. 2
Paul Groult, 1870	Journal	French	*Seccec*	Les parasites extérieurs de l'homme (Suite): p. 6
(*20*)	Thesis	Spanish	*Pique, nigua, huchhuy piqui*	p. 202
(*21*)‡	Journal	English	None	An Ancient Peruvian Effigy vase exhibiting disease of the foot. Plate XLV: p. 730
(*22*)‡	Journal	English	Piquinosis	Utosic syphilis and some other things of interest to paleo-American medicine, as represented on huacos potteries of Old Peru: By Albert S. Ashmead, M.D., of Canadensis, Pennsylvania (First Part). Fig. XIII: p. 336. Idem (Fourth part): p. 490
(*12*)	Journal	English	*Nigua*	New Observations in Paleopathology: p. 246
(*23*)‡	Journal	English	Sandflea, *nigua*	Studies in Paleopathology: The diseases of the Ancient Peruvians, and some account of their surgical practices. Fig. 73-B: p. 216
(*24*)‡	Book	English	Sandflea, *nigua*	Chapter XV. Diseases of Ancient Peruvians. Plate CXIII (c): p. 532–533
(*25*)‡	Journal	Spanish	*Parásitos*	Arte Antiguo Peruano Tecnología y Morfología. Album fotográfico de las principales especies arqueológicas de la Cerámica Muchik existentes en los Museos de Lima. Primera Parte. Tecnología y Morfología: plate 65
(*26*)‡	Book	French	*Piqui, piques*	Chapitre premier. Le Mal et les guérisseurs. Les causes des maladies. Fig. 14: p. 44
(*27*)	Book	Spanish	*Huchuy piqui, nigua, pique*	Volumen 1. La Medicina Incaica. Capitulo XVI. Las Enfermedades: p. 159
(*13*)	Book	English	*Piqui,* sand flea	The Knowledge of parasites. Pre Columbian America - Peru: p.2. Treatment and prevention of parasitic diseases. I. External treatment: p. 212
(*28*)‡	Journal	English	*Nigua,* sand flea, *Tunga penetrans*	The Sandflea –*Tunga penetrans*: pp. 169–176. Representation of parasitic diseases and Parasites in Early African and pre-Columbian Art: II. Early American art. Nigua-Sandflea infection. Tunga penetrans. Plate XVI: pp. 208–209
(*29*)	Bulletin	Spanish	*Niguas*	La Enfermedad en las creencias de los primitivos americanos: p. 28
(*14*)	Book	Spanish	*Niguas*	Nosología precolombina. Parásitos externos. Pulgas: p. 92

*All sources are cited exactly as they appeared on the date of publication and in original languages.
†This ancient and unique manuscript by the indigenous Peruvian chronicler Guaman Poma de Ayala has been digitalized by the Department of Manuscripts and Rare Books, The Royal Library of Denmark, and is available at www.kb.dk/permalink/2006/poma/info/en/frontpage.htm
‡Sources in which reproductions of tungiasis-depicting potteries are available.

Table 2. Characteristics of Incan anthropomorphic vessels depicting tungiasis in Peru

Culture	Historic period*	Where found	Year of first reproduction (reference)	Current location†	Author, year of publication (reference)
Chimu	c. AD 1200–1470	Pachacamac Valley	1907 (*25*)	American Museum of Natural History, New York, NY, USA	Ashmead, 1907 (*21*), Moodie, 1920 (*23*), d'Harcourt, 1939 (*26*)
Chimu	c. AD 1200–1470	Chicama Valley	1924 (*20*)	Museum in Lima, Lima, Peru	Tello, 1924 (*25*), Ashmead, 1910 (*30*)
Maranga	c. AD 150–650	Las Palmas, Surquillo	Never	Museum Foundation Amano, Lima, Peru‡	Weiss, 1984 (*29*)

*Estimated flourishing period of the culture.
†The 2 vessels described by Ashmead, and subsequently reproduced by Moodie and d'Harcourt, and the fragment identified during this study are not publicly exhibited.
‡This piece, no. 1219, is stored with many other vessels that represent diseases of the ancient Peruvians.

his left foot. The handle of the bottle is molded into the shape of a human face. It was found in the Chicama Valley, Ascope, La Libertad (Figure 1, panel B), and its current location is unknown.

The 2 pieces of anthropomorphic pottery shown in Figure 3 also originated from the Chimu culture. They depict 2 men observing the soles of their feet, which show multiple holes of varying sizes. The pieces are located in the American Museum of Natural History but are not on display. They had been found in the Pachacamac Valley, a sandy area in modern southern Lima (Figure 1, panel B).

Figure 2. Chimu culture *huaco* depicting a person extracting parasites with an awl from the sole of the left foot. Multiple holes of various sizes can be seen on the *huaco*.

The anthropomorphic piece shown in Figure 4 originated from the Maranga culture (c. AD 150–650). It is a fragment that portrays a person whose right arm, upper torso, and head are broken off. The left arm and leg are decorated with black, triangle-shaped tattoos arranged in a linear distribution. Cream-colored tweezers hang from the person's chest. The person is using a stick to extract foreign bodies from a cluster of elevated lesions with central holes in the heel of the left foot. There are also 8 holes in the posterior external aspect of the sole, which are clustered and highlighted by a brick-red background. This piece was located in a storage room at the Amano Museum Foundation and, to our knowledge, has not been previously described or reproduced. It was originally found in Las Pampas, Surquillo, Lima (Figure 1, panel B).

Discussion

Tungiasis is an old disease that has been endemic to Peru for centuries and has been illustrated by anthropomorphic pottery showing pathognomonic lesions at various stages of progression. Although the Incas and their ancestors lacked a written language, they used pottery to depict diseases, customs, ceremonies, rituals, and many other activities, thus creating a visual record of their knowledge of a disease process that existed for centuries; such pieces of pottery now provide vivid documentation of their sufferings.

The *huaco* from Chicama Valley (Figure 2) was described by the Harvard-educated Peruvian archeologist Julio C. Tello (1880–1947). Tello was the first indigenous archeologist of America and is considered the father of Peruvian archeology. In 1924, he reproduced this vessel in a collection of 280 pictures of pottery originating from the Mochica (Moche or Muchik) culture titled Arte Antiguo Peruano, volume II; all pieces depicted in this work are distributed among various museums in Lima (*20*). Multiple holes in the left plantar surface of the depicted person, a distinguishing feature of tungiasis, can be observed in this figure. Furthermore, the depicted person holds an awl-like instrument in its right hand, which was then commonly used

Figure 3. Two globular Chimu *huacos* found in Pachacamac, a sandy land area in northern Lima. Each person is examining the soles of the feet, on which multiple punch-out lesions can be detected. Panels B and D are views of the feet of the *huacos* shown in panels A and C, respectively. Catalogs B/8853 and B/8854, courtesy of the Department of Anthropology, American Museum of Natural History.

for removing the parasite from the skin. As a physician, Tello easily recognized these lesions as signs of tungiasis or piquinosis (pique infection) (*22*). However, this collection of pictures of Mochican pottery lacks detailed information about where they were originally found or where they were at the time of its publication. Although it was reproduced as part of a Mochica collection, this *huaco* actually originates from the Chimu culture. Indeed, the Mochica culture (BC 100–AD 700) developed earlier than the Chimu culture (AD 1300–1470); although both cultures flourished in the Moche Valley (Figure 1, panel B), the Chimu culture was a continuation of the Mochica culture (*31*). In other words, the Chimu culture was the same generation as the Mochica culture, but the Chimu culture had a somewhat different ceramic style.

A reproduction of the same *huaco* (Plate 65 in Tello's Mochica collection) was published 14 years earlier, in 1910, by Albert S. Ashmead (1850–1911) (*30*). Ashmead was one of the first North American physicians to study Peruvian potteries that depicted diseases, predominantly leprosy and syphilis, at the beginning of the 20th century (*32*). He received a diverse array of pictures of *huacos* (including that in Figure 2) directly from Tello, with whom he corresponded regularly. With Tello's permission, Ashmead subsequently published his reproduction of this *huaco* (*22*). Unlike Tello, Ashmead documented the site at which this *huaco* was originally found, the Chicama Valley (this information was probably provided by the archeologist who discovered the piece). Nevertheless, Ashmead did not associate these lesions with tungiasis and instead thought they were a product of syphilis. In a letter addressed to Ashmead, Tello uses the word piquinosis to describe to the tungiasis depicted on *huaco* 1; unfortunately, Ashmead did not recognize this regional term (piquinosis = pique infection) used to designate tungiasis (*22*).

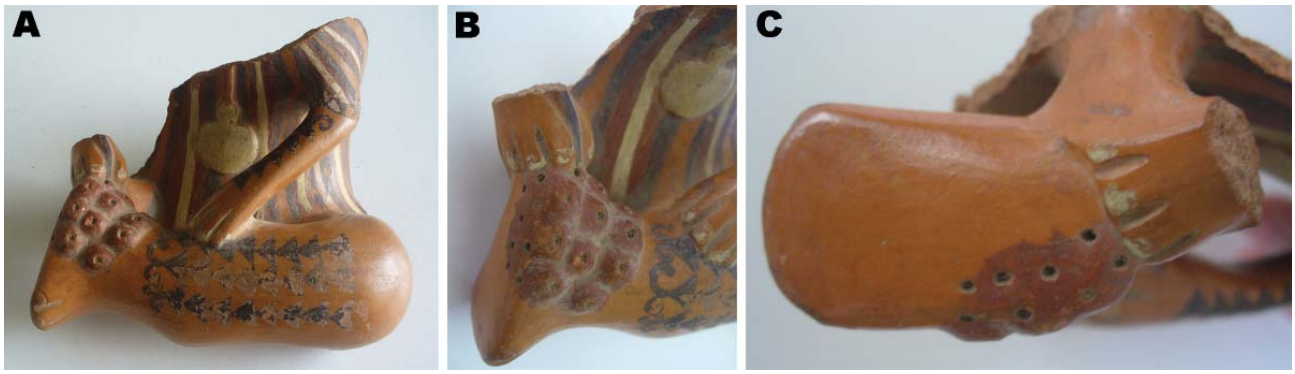

Figure 4. A) Polychromic Maranga culture fragment that portrays a torso and a tattooed left leg of a person holding a stick while extracting foreign bodies. Cluster lesions with elevated nodules and a central black depression suggest *Tunga* spp. infection. B) Closer view of the left heel. C) Details of the sole of the left foot, showing multiple holes over a brick-red surface, suggesting residual tungiasis lesions. No. 1219, courtesy of the Amano Museum Foundation.

The 2 *huacos* from the Pachacamac Valley (Figure 3) were first published by Ashmead in 1907 (*21*). As with the *huaco* from the Chicama Valley (Figure 2), Ashmead erroneously concluded that the lesions depicted on the soles of the feet of persons depicted on these 2 jars represented signs of *uta*, which he believed to be skin tuberculosis (the etiology of *uta*, or cutaneous leishmaniasis, in Peru was later unveiled during the 1913 Harvard expedition to the Amazon region led by Richard P. Strong (*33*). Because he was interested in prehistoric syphilis and Peruvian earthenware representing diseases, Ashmead maintained correspondence with several renowned physicians from Lima, including Tello. However, Ashmead never associated these 2 *huacos* with tungiasis, arguing that the holes in the feet were too prominent to represent tungiasis (*32*). It was paleopathologist Roy L. Moodie (1880–1934) and then Americanist Raoul d'Harcourt (1879–1971) who later reevaluated the significance of these vessels, both concurring that the holes on the feet of these 2 *huacos* represent residual lesions left by *nigua* infections (*12*,*23*,*24*,*26*). Pachacamac, the site at which these 2 jars were located, was not part of the Chimu culture's territory (Figure 1, panel B). Because the Old Sanctuary of Pachacamac was the major place of worship of the pre-Hispanic Peruvian coast for >1,500 years (*31*), its temples were visited by masses of pilgrims from the entire Andean world, who carried with them diverse offerings, including *huacos*, during religious rituals and ceremonies. Thus, archeological pieces from the coastal, highland and Amazon regions of Peru can be found in Pachacamac.

During our visit to the Amano Museum Foundation in 2009, we found the fragment of a *huaco* from Las Palmas (Figure 4) in a private collection storage room. It had originally been excavated by Yoshitaro Amano (1898–1982), a prosperous Japanese businessman who arrived in Peru in 1951 and was captivated by its history. He excavated and rescued innumerable pieces from sacked and abandoned archeological sites. Pedro Weiss (1893–1985), a Peruvian pathologist who dedicated part of his life to the study of these potteries, mentioned that there were representations of *niguas* in this museum in his 1980 article La Enfermedad en las Creencias de los Primitivos Americanos; however, he neither photographed nor described any *huacos* (*29*). In contrast to the evidence we have for the previously described *huacos*, we do not have strong evidence proving that this fragment was the one described by Weiss in his above-mentioned work. Together with the first 3 vessels described here, which were also cited by Hoeppli in 1959 as early documentation of parasites in the Western Hemisphere (*28*), to our knowledge, this fragment is the fourth representation of *Tunga* spp. infection identified in pre-Hispanic American art. Furthermore, it is the only vessel that depicts different stages of tungiasis, thus representing explicit evidence of its endemicity in ancient Peru.

Along with these 4 *huacos*, additional evidence suggests the presence of tungiasis in pre-Incan Peru. The 2 most common names attributed to the sand flea in Peru and other countries of South America— *nigua* and *pique*—come from the Arawak and Quechua languages, respectively. Indeed, Quechua was the official language of the Incan Empire and is currently the second most commonly spoken language in Peru, after Spanish. Furthermore, the Incas named it *seccec* from the verb *seccen*, a Quechua word that means itching (*16*,*17*). Currently, it is called *huchuy piqui* (or *huchhuy piqui*, according to Lavoveria [*20*]) or *ushtuchi piki* by Amerindian communities in the Highlands.

Another aspect of pre-Incan tungiasis is documentation of the therapeutic approaches by historians, anthropologists, and physicians. For example, in his book La Médecine dans l'Ancien Pérou, d'Harcourt mentioned that Peruvian natives used a stick to remove fleas from their feet (*26*), similar to what is observed on our fragment. In addition, Lastres, in his compendious Historia de la Medicina Peruana, mentioned *nigua* as being endemic to Peru and described the application of sweet potatoes leaves to the feet to treat infections (*27*).

Until now, numerous factors have impeded our understanding of the history of tungiasis in Peru. First, the sand flea has been given multiple names by populations living in parasite-endemic areas, making literature searches difficult. *Nigua, pique, jigger, chigoe, puce-chique,* and *tchique* are only a few of the many names that have been given to this burrowing flea. Second, it has been taxonomically reclassified multiple times with different names by entomologists over the past 3 centuries (*16*–*19*,*34*). Finally, the high rates of endemicity, along with a relatively uncomplicated clinical course, have made it a disease that is underreported and neglected among physicians in Peru (*8*).

Our search had some limitations. The dispersed distribution of these Peruvian anthropomorphic pieces in art museums throughout the world made it difficult to document the exact number of pottery pieces that depict tungiasis (*35*). An unknown number of disease-illustrating *huacos* remain to be located and investigated. At the beginning of the Spanish Conquest, the conquerors looted religious places in their quest for gold, leaving behind innumerable pieces of pottery made by the Incas and their predecessors. Later, at the beginning of the 20th century, theories about the people of the Americas were propounded along with the study of pre-Hispanic cultures. As a result, sacred places, ceremonial paraphernalia, and other anthropologic pieces in the coast and the Andes were unearthed. These clay pottery pieces were deemed rarities and were highly prized by antiquity collectors. In fact, Ashmead and Tello clearly

stated that a large number of Peruvian archeological pieces were highly prized on the black market in their time (*36*, *37*). Even today, substantial illicit traffic of ceramics from ancient Peru continues, which has forced the International Council of Museums to include Mochica vessels in the Red List of Latin American Cultural Objects at Risk (*38*).

Our photograph of the newly identified fragment depicting tungiasis provides additional evidence of tungiasis among ancient Peruvians. The knowledge of this disease in pre-Incan cultures is a valuable legacy that gives a historical insight into the endemicity of this arthropod in South America. These anthropological pieces are now dispersed among numerous museums worldwide. Their identification and analytic evaluation is critical for enhancing our understanding of the history and effects of this flea that continues to affect Peruvians today as it did in pre-Incan times.

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Human Intraocular Filariasis Caused by *Dirofilaria* sp. Nematode, Brazil

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A case of human intraocular dirofilariasis is reported from northern Brazil. The nematode was morphologically and phylogenetically related to *Dirofilaria immitis* but distinct from reference sequences, including those of *D. immitis* infesting dogs in the same area. A zoonotic *Dirofilaria* species infesting wild mammals in Brazil and its implications are discussed.

Zoonotic filariases are caused by nematodes of the superfamily Filarioidea and are transmitted by blood-feeding arthropods. Within this taxonomic group, nematodes of the genus *Dirofilaria* are among the most common agents infecting humans (*1*–*5*). The 2 main species of zoonotic concern are *Dirofilaria immitis*, which causes canine cardiopulmonary disease, and *D. repens*, which is usually found in subcutaneous tissues. In addition, zoonotic subcutaneous infections with *D. tenuis* in raccoons and *D. ursi* in bears have been reported less frequently in North America (*3*). *Dirofilaria* spp. infections in humans have been detected mostly in subcutaneous tissue and lungs (*3*), and 1 intraocular case of infection with *D. repens* was reported from Russia (*1*). *D. immitis* and *D. repens* are the main causes of human dirofilariasis in the Americas (*4*,*6*) and Old World (*2*,*5*), respectively.

Morphologic identification of *Dirofilaria* spp. is based on the body cuticle, which is smooth in *D. immitis* (subgenus *Dirofilaria*) and has external longitudinal ridges in *D. repens* and other species of the subgenus *Nochtiella*. However, in many reported cases of zoonotic dirofilariasis, specific identification was not adequately addressed (*5*). Twenty-eight cases of human dirofilariasis in the Old World attributed to *D. immitis* have been reviewed recently and attributed to *D. repens* (*5*). On the basis of this information, the suggestion was made that *D. immitis* populations have different infective capabilities for humans in the Old and New Worlds (*5*). However, this hypothesis was not supported by recent genetic comparisons of specimens from the Old World (Italy and Japan) and New World (United States and Cuba) (*7*; M. Casiraghi, unpub. data).

We report a case of human intraocular dirofilariasis in which a live male *Dirofilaria* sp. worm was recovered from the anterior chamber of a patient's eye. Morphologic and molecular studies suggested that this zoonotic case of dirofilariasis was caused by a *Dirofilaria* sp. closely related to *D. immitis*.

The Study

On September 16, 2008, a 16-year-old boy came to the Clínica de Olhos do Pará in Pará, Brazil, with low visual acuity (0.54 m), an intraocular pressure of 44 mm Hg, and pain in the left eye. Ophthalmologic examination showed a nematode (online Appendix Video, www.cdc.gov/EID/content/17/5/863-appV.htm) in the anterior chamber of the left eye. The patient reported no travel history in recent months preceding the onset of symptoms. Corneal edema (Figure 1, panel A) and episcleral hyperemia in the left eye were observed, and surgery for removal of the nematode was recommended. After peribulbar anesthesia, the eye was clipped and the cornea was incised with a crescent Beaver corneal knife, and the nematode was extracted with forceps and Fukasacu hook (online Appendix Video). A live filarial nematode was removed from the anterior eye chamber. The patient recovered without complications after surgery.

The parasite (deposited at the Muséum National d'Histoire Naturelle, Parasitologie Comparée, Paris, France; accession no. 143 YU) was a male filarial nematode (length 106 mm, width 400 μm). It had large caudal alae, several pairs of pedunculated papillae (Figure 1, panel B), unequal spicules, a short and thick tail, and an esophagus with a wider glandular region.

Narrow hypodermal lateral chords and the 2 lateral internal cuticular crests are typical for *Dirofilaria* spp. (*8*). The specimen was not *D. repens* or a *Nochtiella* species because of the smooth body cuticle. The left spicule (length 378 μm), with a handle and lamina of equal length, was similar to that of *D. immitis* (Figure 1, panel C). Other measurements were similar to those of *D. immitis* (esophagus length 1,200 μm; right spicule length 170 μm; tail length 110 μm; width at the anus 110 μm) (*8*).

To confirm morphologic identification, a DNA barcoding approach was used (*7*). A small piece (≈3 mm) of the nematode was used for DNA extraction and

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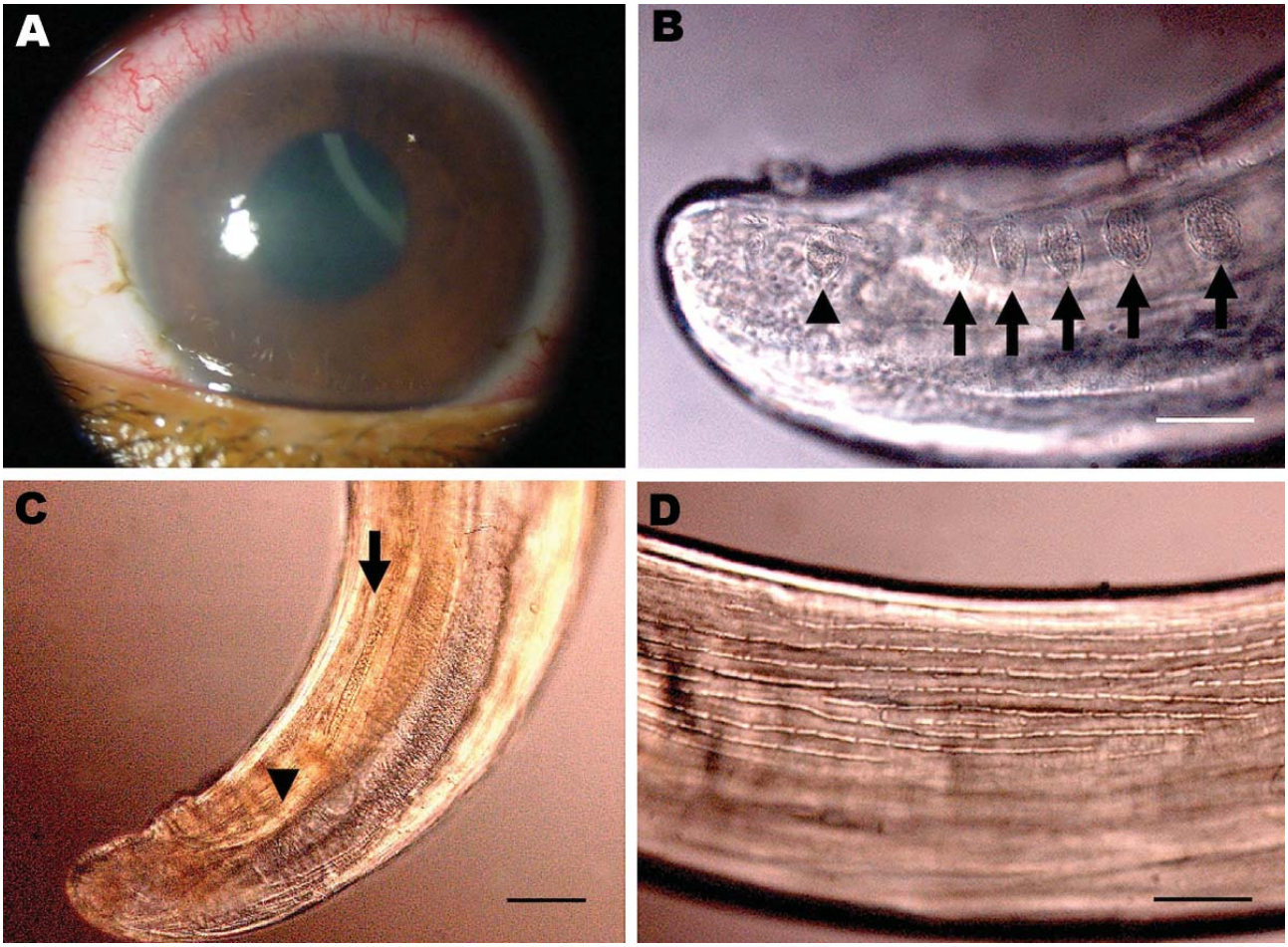

Figure 1. Corneal edema and episcleral hyperemia in the left eye of a 16-year-old boy from Brazil and a free-swimming filarid in the anterior chamber. A) Macroscopic view. B) Five pairs of ovoid pre-cloacal papillae (arrows) and 1 postcloacal caudal papillae (arrowhead). Scale bar = 50 µm. C) Small (arrowhead) and large (arrow) spicules. Scale bar = 40 µm. D) Longitudinal ridges of the area rugosa. Scale bar = 50 µm.

amplification of cytochrome c oxidase subunit 1 (*cox1*) and 12S rDNA gene fragments as described (*9*). Sequences were deposited in the European Molecular Biology Laboratory Nucleotide Sequence Database (accessions nos. HQ540423 and HQ540424). In accordance with morphologic identification, BLAST (http://blast.ncbi.nlm.nih.gov) analysis of both genes showed overall highest nucleotide similarity with those of *D. immitis* available in GenBank (12S rDNA, accession nos. AM779770 and AM779771; *cox1*, accession nos. AM749226 and AM749229).

Phylogenetic analysis using *cox1* sequences by MEGA 4.0 (www.megasoftware.net), a neighbor-joining algorithm, and Kimura 2-parameter correction confirmed that 143 YU clustered with *D. immitis* from different countries, including Australia (GenBank accession no. DQ358815) and Italy (other sequences in Figure 2). The *cox1* sequence of *D. immitis* from a dog in Pará, Brazil, was identical with those of the same species in GenBank. Topology of 12S rDNA sequences was identical with that of *cox*1 sequences. However, comparison of nematode sequences with those in GenBank showed large differences in nucleotide similarities (5% and 6% for 12S rDNA and *cox1*, respectively).

Morphologic comparison with male worms isolated from dogs (1 from People's Republic of China and 1 from Japan, Muséum National d'Histoire Naturelle accession nos. 63 SE and 169 YU) showed that the area rugosa (ventral ornamentation of the posterior region) was similar among all specimens examined and was composed of ≈8 longitudinal crests, each containing aligned segments (length 15–30 µm) extending 1–8 mm from the tail tip (Figure 1, panel D). Arrangement and number of caudal papillae were also similar (*10*). The preesophageal cuticular ring was present in *D. immitis* reference specimens but was not present in the specimen from Brazil. In addition, the deirids were more anterior in the worm from Brazil than in

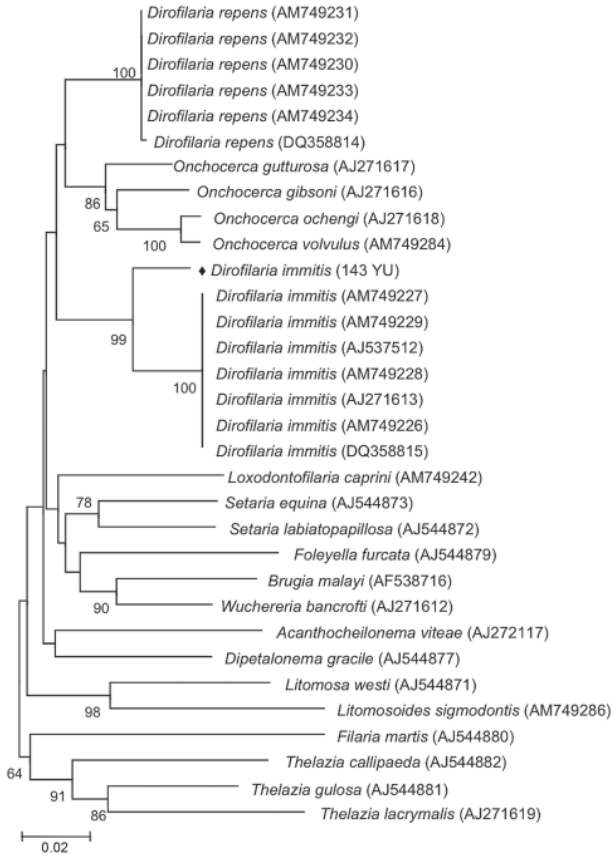

Figure 2. Phylogeny of filarial nematodes based on cytochrome c oxidase subunit 1 (*cox1*) gene sequences. *Thelazia* spp. species were used as outgroup. Bootstrap confidence values (100 replicates) are shown at the nodes only for values >50%. Solid diamond indicates nematode isolated in this study. Numbers in parentheses are GenBank accession numbers. Scale bar indicates nucleotide substitutions per site.

reference specimens. This finding might be caused by less growth of the nematode found in the patient from Brazil.

Similar morphologic features (small size and anterior deirids) have been reported in a *D. immitis* male worm from a dog in Cayenne, French Guiana (*11*). However, a *D. spectans* worm from an otter in Brazil had a similar body size and location of deirids as the worm 143 YU but a different pattern of juxtacloacal papillae.

Conclusions

In Brazil, human pulmonary dirofilariasis caused by *D. immitis* has been reported sporadically (*12*), mainly in southeastern Brazil, where the prevalence of heartworm in dogs ranges from 2.2% to 52.5% (*13*). The patient in this study came from a region in Brazil where canine dirofilariasis caused by *D. immitis* is endemic and in which the prevalence of microfilaremic dogs is ≤32.5% (*14*). Morphologic features and overall high identity of 12S rDNA and *cox1* gene sequences compared with the reference DNA sequences confirmed that the worm recovered was similar to *D. immitis*. However, nucleotide similarity differences in comparison with other sequences of *D. immitis* available in GenBank (including 1 from Brazil) were 5% and 6% for 12S rDNA and *cox1* genes, respectively, which are higher than the range of intraspecific variation (<0.8%) reported for *D. immitis* originating from the United States, Italy, and Japan (*7*). Such variation (>5%) has not been reported for a *Dirofilaria* species. Therefore, existence of a closely related species (e.g., *D. spectans* from otters, but also reported from a human in Brazil) (*15*) should be considered. Further investigations of filarial nematodes from dogs and wild mammals in Brazil are required to elucidate the identity of this *Dirofilaria* species and its primary hosts and vectors.

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Human Intraocular Filariasis Caused by *Pelecitus* sp. Nematode, Brazil

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A male nematode was extracted from iris fibers of a man from the Brazilian Amazon region. This nematode belonged to the genus *Pelecitus* but was distinct from the 16 known species in this genus. Similarities with *Pelecitus* spp. from neotropical birds suggested an avian origin for this species.

Filarial nematodes have been found in the eyes and periorbital region of humans worldwide (*1*–*4*). However, rarely have the worms been removed and morphologically described. The main human filarial parasites are *Wuchereria bancrofti* and *Brugia malayi*, whose adults live in the lymphatic system, and *Loa loa*, which infects subcutaneous tissues. In addition, some filarioids have an animal origin, either from domestic mammals, such as for *Dirofilaria* spp., or from wild mammals, including *Onchocerca*, *Molinema*, and *Loaina* spp. (*1*,*2*,*5*).

Nematode identification at the species level might be supported by anamnestic information, such as host and geographic location. However, for a reliable, definitive, species identification, proper morphologic or molecular diagnosis is needed. Clinical reports may provide a useful database for better understanding of the zoonotic potential of little-known filarioids infecting wild animals. We report a case of human intraocular filariasis caused by a *Pelecitus* sp., briefly describe the main morphologic features for nematode identification, and suggest the origin of this zoonotic infection.

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The Study

On August 2007, a 29-year-old man from Tucuruí in northern Brazil, who worked in power grid maintenance in a forested area, came to his ophthalmologist with an intraocular larva in the left eye. There was no familial history of ophthalmologic disorders, and ophthalmologic examinations showed that the patient had visual acuity and corrected vision of 20/25 in both eyes. Biomicroscopy showed a transparent cornea in the right eye without lesions or edema, an anterior cavity without an inflammatory reaction, and an anterior subcapsular cataract of +/4+. The cornea in the left eye was transparent and did not have lesions or edema, and the anterior chamber did not show an inflammatory reaction. No funduscopic alterations were found in either eye by direct and indirect ophthalmoscopic examination.

An ≈4-mm worm with undulating movements was observed between the muscular fibers of the iris (Figure 1, panel A). The patient underwent surgery 1 day after the consultation, and he consented to the publication of this clinical case. After peribulbar anesthesia, a 2-mm corneal incision was made at the 11 o'clock position. The nematode was extracted by aspiration (online Appendix Video, www.cdc.gov/EID/content/17/5/867-appV.htm) and placed in saline solution. No surgical complications occurred (Figure 1, panel B), and the patient did not have ocular symptoms during the 6 months after surgery.

The worm was fixed in 2% acetic acid, 3% formaldehyde, and 95% ethanol; mounted in glycerine jelly; and later transferred into lactophenol. The specimen was preserved in absolute alcohol at the National Museum of Natural History (Paris, France) (accession no. 138 YU). This male nematode (length 4.5 mm, width 300 μm at mid-body) had a coiled and twisted body that tapered at both extremities (Figure 2, panel A). The cuticle (thickness 6 μm) showed 2 rounded, lateral, cuticular alae (thickness 20 μm) along the body and postdeirids 530 μm from the posterior extremity (Figure 2, panel B). The head was bluntly rounded and contained 4 externolabial papillae, 4 cephalic papillae, 2 amphids, and a buccal cavity (length 5 μm, width 4.5 μm) with a tiny cuticular ring. The nerve ring was 165 μm from the anterior end. The esophagus was 765 μm long, increased slightly in diameter in the posterior half, and did not have a distinct glandular part. The large caudal alae had 2 granular inclusions on each lateral side. The tail was 48 μm long. Five pairs of caudal papillae (2 pedunculated, precloacal, lateral; 1 small subventral closely posterior to the cloacal opening; and 2 pedunculated lateral pairs on posterior half of the tail) were observed, and the 2 phasmids were subterminal. The 2 spicules (length 66 μm

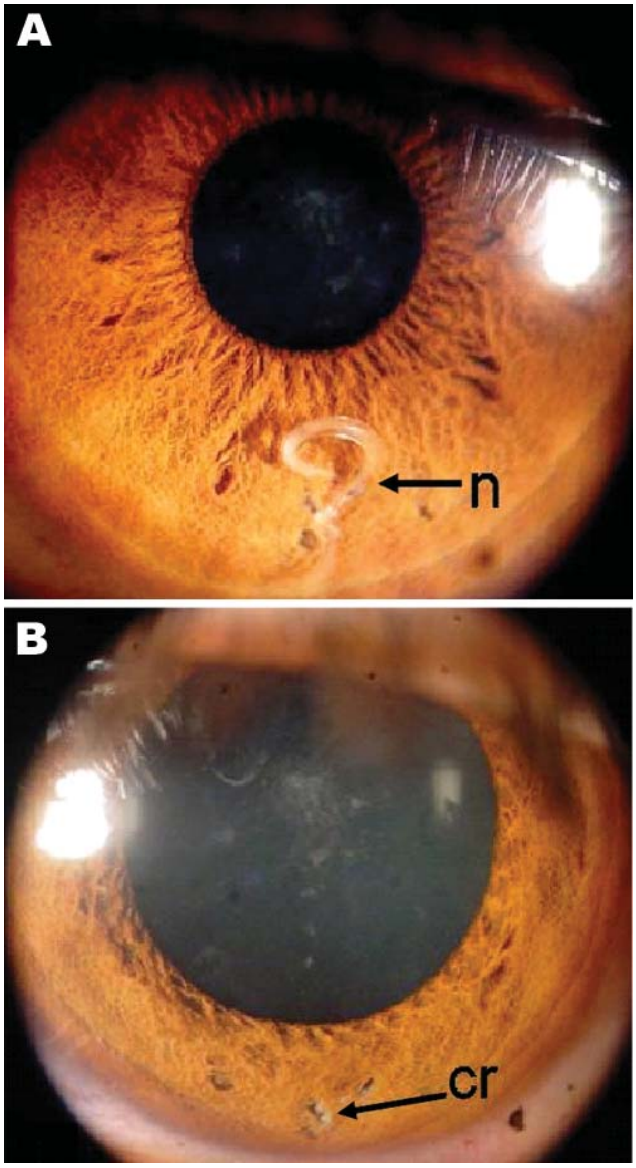

Figure 1. Eye of the patient, a 29-year-old man from Brazil. A) Nematode (n) between muscle fibers of the iris. B) Iris after surgery, showing a mild residual scar (cr) in the region where the nematode had been located.

and 82 μm) (Figure 2, panels C, D) were dissimilar. The larger left spicule had a typical beveled extremity.

Many morphologic characteristics of the filarial worm resembled those of *Pelecitus* spp. (*6*) (coiled and twisted body that was attenuated at both extremities; lateral alae from the cervical region to distal tip of body; postdierids within alae in the posterior half of body; and a delicate, preesophageal, cuticular ring). *Pelecitus* spp. include mainly parasites of birds and a few mammals, some of which have been identified as *Loaina* spp. (*6*,*7*).

The specimen from the patient was compared with the 16 known species of *Pelecitus* described (*6*,*8*,*9*), but the specimen did not match any of them. The worm differed from the only 2 species found in lagomorphs (*P. scapiceps* and *P. meridionaleporinus*) (*9*) in North America and Mexico, which had a beveled extremity on the right spicule (*8*,*9*) instead of the left spicule.

A small male filaria was recovered from the anterior chamber of a human eye in Colombia (*5*). This filaria was originally assigned to the genus *Loaina* but was later identified as a species of *Pelecitus* (*6*). Like the nematode specimen we describe, it was morphologically similar to some species that infect birds.

Conclusions

We assigned the worm found in the anterior chamber of the eye of the patient to the genus *Pelecitus* (*6*). The species of *Pelecitus* that infected the eyes of 2 humans (reported here and in Colombia) (*5*) remains unidentified. These cases were found in the tropical Amazon region (Pará, Brazil) and the Department of Antioquia (northwestern Colombia). The male specimen of *Pelecitus* sp. described here and the species that infected a human in Colombia are similar but distinct. However, in both cases, a mammalian origin of these zoonotic agents seems unlikely because of differences identified by comparing these worms with parasitic species infecting lagomorphs (*8*,*9*). Both cases of human infection with *Pelecitus* spp. more likely have an avian origin. Vectors of *Pelecitus* spp. are mosquitoes, chewing lice, and tabanids, as shown with the 3 cycles elucidated (*10*–*12*).

Although infection of birds in South America by *Pelecitus* spp. has been reported (*13*,*14*), information on this taxon is scant. Many nematode species have not been identified because of lack of basic information on filarial fauna of animals. This dearth of information is particularly true for regions, such as the Amazon rain forest in Brazil, where wide biodiversity and many unidentified animal and plant species are found (*15*). Consequently, species identification of filarioid nematodes that infect human eyes is difficult if not impossible. However, our identification of this filarid should help clarify the zoonotic role of filarioid infections in humans in tropical regions and increase awareness of physicians and ophthalmologists of the variety of nematodes that may be found in the human eye.

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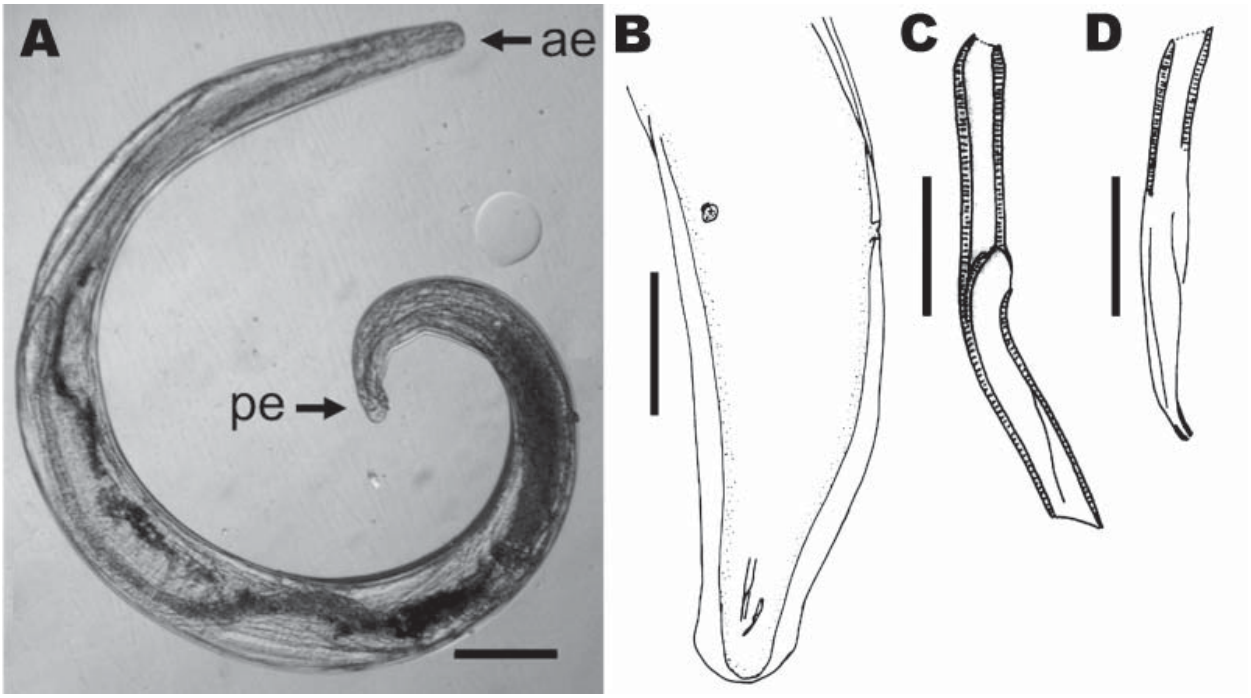

Figure 2. Parasitic nematode isolated from the eye of the patient, a 29-year-old man from Brazil. A) Nematode that was removed from the iris, showing anterior (ae) and posterior (pe) extremities. Scale bar = 200 µm. B) Caudal region, subdorsal view, showing lateral alae, spicules, and the 2 postdeirids. Scale bar = 150 µm. C) Left spicule; D) right spicule. Scale bars = 20 µm.

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Linguatula serrata Tongue Worm in Human Eye, Austria

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Linguatula serrata, the so-called tongue worm, is a worm-like, bloodsucking parasite belonging to the Pentastomida group. Infections with *L. serrata* tongue worms are rare in Europe. We describe a case of ocular linguatulosis in central Europe and provide molecular data on *L. serrata* tongue worms.

The species *Linguatula serrata* belongs to the Pentastomida, a still-enigmatic group of worm-like, bloodsucking parasites that inhabit the upper respiratory tract of terrestrial, carnivorous vertebrates, mostly reptiles and birds; *L. serrata*, commonly called tongue worms, typically inhabit canids and felids. The intermediate hosts of these parasites are usually sheep, cattle, or rodents. The hosts ingest the eggs, and the first instar larva hatches within their intestines, penetrates the mucosa, and retreats into the tissue, where it encysts and molts to the third larval stage. Humans can serve as aberrant final hosts after ingesting raw or poorly cooked viscera (i.e., liver, lungs, and trachea) of intermediate hosts. This nasopharyngeal infection is known as Halzoun syndrome in the Middle East or as Marrara in Sudan (*1*,*2*). Humans can also serve as accidental intermediate hosts, when ingesting the eggs (visceral infection) (*3*). Intraocular infection is extremely rare; only 5 cases caused by *L. serrata* tongue worms have been described: 2 from the southern United States (*4*–*5*), 1 from Portugal (*6*), 1 from Israel (*7*), and 1 from Ecuador (*8*).

The phylogenetic position of the pentastomids is still not fully resolved. A position as the sister group of branchiurian crustaceans (Maxillopoda) is supported by molecular data and sperm morphology (*9*–*11*) and is widely accepted today; however, sound evidence also exists for other classifications (*12*). The group itself is divided into 2 orders, the more primitive Cephalobaenida and the more advanced Porocephalida. All species infecting humans are currently classified as Porocephalida; the species *L. serrata* and *Armillifer armillatus* are responsible for most human cases of infection.

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The Study

A 14-year-old girl was referred to the eye clinic at the Medical University of Vienna with an unknown parasite detected during ophthalmologic examination. The girl had redness, pain, and progressive visual loss in the right eye. Her medical history was unremarkable except that she had reported regular contact with domestic animals: 2 dogs, cats, and 1 turtle. She had no history of bites or other infestations, and neither she nor any of her animals had been abroad.

Vision was reduced to 0.1 Snellen. Slit lamp examination revealed a mobile parasite swimming like a fish in the anterior chamber of the eye (online Appendix Video, www.cdc.gov/EID/content/17/5/870-appV.htm); signs of local inflammation with cells and Tyndall phenomena were present. The pupil was round and reactive, the lens was clear, and a slight iridodonesis was observed. Fundus examination through a constricted pupil showed no abnormalities. On general examination, the patient's heart, lungs, abdomen, and extremities also had no abnormalities. Her nose and throat were examined carefully for additional parasites. Serologic data including serum chemistry, C-reactive protein, fasting blood glucose, erythrocyte sedimentation rate, creatinine, and blood urea nitrogen levels were within normal limits. Her complete blood count revealed cell differentiation within normal limits without eosinophilia. Her chest and sinus radiographs showed no abnormalities. Surgical removal of the parasite was complicated because of high mobility of the parasite inside the anterior chamber. The worm escaped into the posterior segment of the eye where it was found, after lens removal and complete vitrectomy, in a recess of the ciliary body. A viable parasite was extracted and transferred to physiologic saline. One month later, the eye was completely free of irritation, and 3 months later an artificial intraocular lens (ARTISAN; OPHTEC BV, Groningen, the Netherlands) was implanted. Final visual acuity was 1.0 Snellen.

The surgically extracted parasite was examined microscopically and then subjected to DNA isolation with the QIAGEN tissue kit (QIAGEN, Hilden, Germany). The 18S rDNA was amplified and sequenced stepwise by using 6 internal primers (P1fw, P1rev, P2fw, P2rev, P3fw, P3rev) (*13*) and a 310 ABI PRISM automated sequencer (Applied Biosystems, Darmstadt, Germany); 3 sequences each were obtained from both strands. Fragments were combined to a consensus sequence with the GENEDOC sequence editor (www.nrbsc.org/gfx/genedoc), and deposited in GenBank (accession no. FJ528908). For cluster analysis, primer sites,

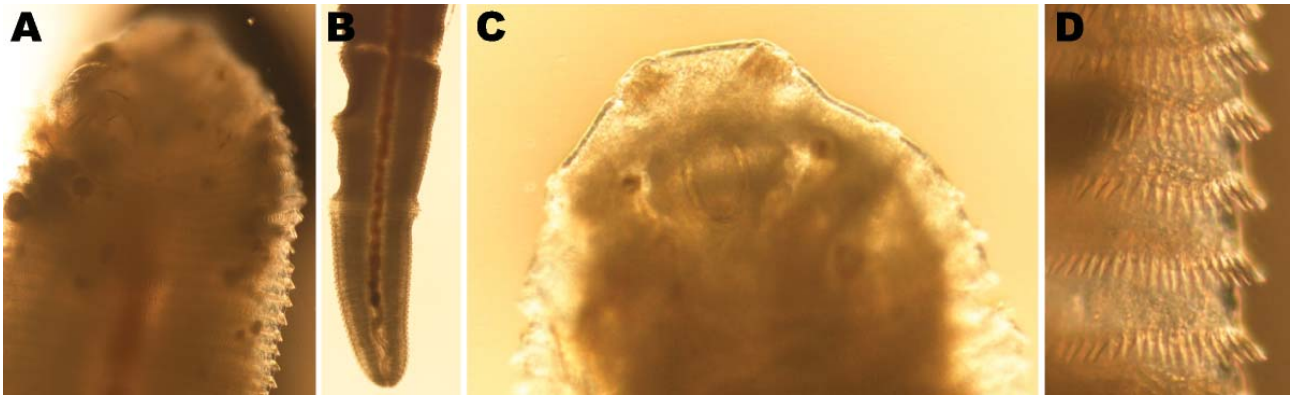

Figure 1. Morphology of *Linguatula serrata* tongue worm. A) Ventral anterior end with hooks. B) Posterior end laterally with primordial uterus, genital porus, and intestine (note peristalsis). C) Dorsal anterior end with cuticular structures of apical papillae, chitinoid oral clasp, and insertions of oral muscles. D) Rows of spicules. Original magnification ×50 (A–C) or ×100 (D) .

unique gaps, and ambiguously aligned sites were excluded, resulting in a dataset of≈1,560 aligned sites. Cluster analysis was performed by using the PHYLIP 3.63 package (www.phylip.com), with neighbor joining, maximum parsimony, and maximum likelihood as evolutionary models and 100 bootstrap replicates generated for each model. Trees were rooted with 4 branchiurian sequences (GenBank nos. DQ925818, DQ925819, AF436004, and DQ925842).

The parasite was 4.5 mm long, appeared whitish, and had a flattened (tongue-like) body shape with a rounded anterior (0.9 mm wide) and pointed posterior end (0.35 mm). It had curved hooks with sharp tips on the anterior ventral side (Figure 1, panel A), a red primordial uterus stretching from the anterior to the posterior end, a median ventral genital porus, and a terminal anus (Figure 1, panel B). The anterior end had apical papillae and a chitinoid oral clasp (Figure 1, panel C). The cuticle showed 94 rings and conspicuous spicules (Figure 1, panel D). Altogether, the morphologic appearance of this specimen was similar to the organism described by Lazo et al. (*8*) and could thus be identified as a third instar larva of *L. serrata* tongue worm. The cuticular rings and the spicules at the posterior margins of the cuticle rings are also characteristic for this parasite (*14*).

We assumed the mode of infection to be ingestion or direct eye contact with *L. serrata* eggs and the source of infection to be 1 of the patient's 2 pet dogs. Dogs are the typical final hosts of *L. serrata* tongue worms, and children generally tend to have carefree contact with pet animals. Unfortunately, neither dog could be investigated for potential infection. Infection rates of dogs in Austria are unknown; only 1 case has been reported, and it occured in a dog imported from the United States (*15*). The girl also had cats and a turtle, but cats are not primary hosts and turtles are never hosts for the life cycle of the *L. serrata* tongue worm (*14*). Two of the other 5 ocular linguatulosis case-patients mentioned earlier had a recent history of ocular trauma but whether trauma was related to infection

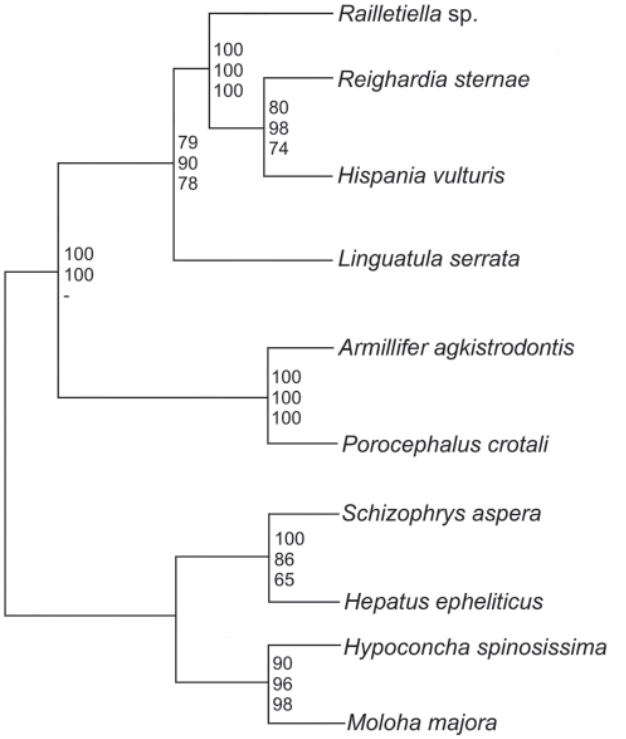

Figure 2. Rectangular cladogram based on 18S rRNA gene sequences of 6 pentastomid species. The tree was rooted with 4 branchiura sequences as an outgroup. The numbers at nodes represent bootstrap values based on 100 replicates (neighbor joining/maximum likelihood/maximum parsimony). Both subgroups of the Pentastomida, the Cephalobaenida (*Reighardia sternae, Hispania vulturis*, and *Raillietiella* spp.) and the Porocephalidae (*Porocephalus crotali* and *Armillifer agkistrodontis*) are well supported. The choice of outgroup had no effect on tree topology but did in some cases reduce bootstrap values.

remains unknown. In the case reported here, no eye injury was noted.

The 18S rRNA gene of *L. serrata* is 1,834 bp long with a GC content of 49.3%. Sequence similarities to other pentastomids range from 90.0% (*Armillifer agkistrodontis, Porocephalus crotali*) to 90.8% (*Raillietiella* spp.). However, the distance between *L. serrata* and the other species is larger than that between any other species. Cluster analyses resulted in homologous consensus trees independent of the evolutionary model used (Figure 2). Notably, *L. serrata* grouped with the Cephalobaenida, supported by high bootstrap values, although in traditional classification it is assigned to the Porocephalida. To date only 5 other 18S rDNA sequences of pentastomids are available, and the sequence of *P. crotali* is incomplete, limiting the basis for cluster analysis to only 1,560 aligned sites. Even when a cladogram without *P. crotali* and on a basis of 1,780 aligned sites was constructed, *L. serrata* remained in the Cephalobaenida. In fact, at least 1 morphologic feature might support this position: typical Porocephalida, such as those in the genus *Armillifer*, have their hooks horizontally lined up, whereas the hooks of cephalobaenids are pairwise obliquely arranged, as in those in the genus *Linguatula* (*14*).

Conclusions

This study indicates that rearrangements in the current classification scheme of the Pentastomida might be necessary. We hope that our data will be an impetus for a comprehensive phylogenetic study of this group of parasites.

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Rickettsia rickettsii Transmission by a Lone Star Tick, North Carolina

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Only indirect or circumstantial evidence has been published to support transmission of *Rickettsia rickettsii* by *Amblyomma americanum* (lone star) ticks in North America. This study provides molecular evidence that *A. americanum* ticks can function, although most likely infrequently, as vectors of Rocky Mountain spotted fever for humans.

Historically, transmission of *Rickettsia rickettsii* has been attributed to *Dermacentor variabilis* ticks in the eastern United States and to *D. andersoni* ticks in the western United States (*1*). Recently, researchers at the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) documented transmission of *R. rickettsii* to persons residing in Arizona by a novel tick vector for North America, *Rhipicephalus sanguineus* (commonly referred to as the brown dog tick or kennel tick) (*2*,*3*). As reviewed by Goddard and Varela-Stokes in 2009, only scant evidence has been published to support transmission of *R. rickettsii* by *Amblyomma americanum* (lone star) ticks in North America (*4*), although Parker et al. in 1933 reported experimental transmission of rickettsial infection by lone star ticks (*1*), and other *Amblyomma* species, such as *A. imitator* and *A. cajennense*, are likely vectors for Rocky Mountain spotted fever (RMSF) in Central and South America (*5*).

The Study

A 61-year-old man visited his physician in May 2010 and reported a history of fevers, myalgias, and headache. He had been free of symptoms until 3 days earlier, when he experienced nausea and dizziness. The following morning, he had chills and, later the same day, a fever of 38.4°C. Mild headache and myalgias accompanied his fever. His symptoms became progressively more severe during the next 2 days, and fever reached 39.4°C.

The patient resided on a farm in central North Carolina and had occasional exposure to ticks. Seven days before the onset of illness, he had removed an embedded tick from his right axilla. He estimated that the tick had been attached for 2–3 days. Clinical examination showed an eschar-like lesion at the site of the tick bite that appeared erythematous with a necrotic center (Figure). The patient's neutrophil count (2,553 cells/μL) was within low reference limits, accompanied by 5% band neutrophils, and mild thrombocytopenia (148,000 platelets/μL).

A tick-borne rickettsiosis or ehrlichiosis was suspected, and on the 4th day of illness, the patient was prescribed doxycycline, 100 mg 2×/d for 7 days. A maculopapular rash appeared the next morning, which predominantly involved the extremities but not the palms or soles. Fever resolved within 36 hours after beginning therapy, and the rash began to fade. Within 1 week after receiving doxycycline, the patient's symptoms and hematologic abnormalities had resolved (neutrophil count 4,378 cells/μL, no band neutrophils, and platelet count of 320,000/μL).

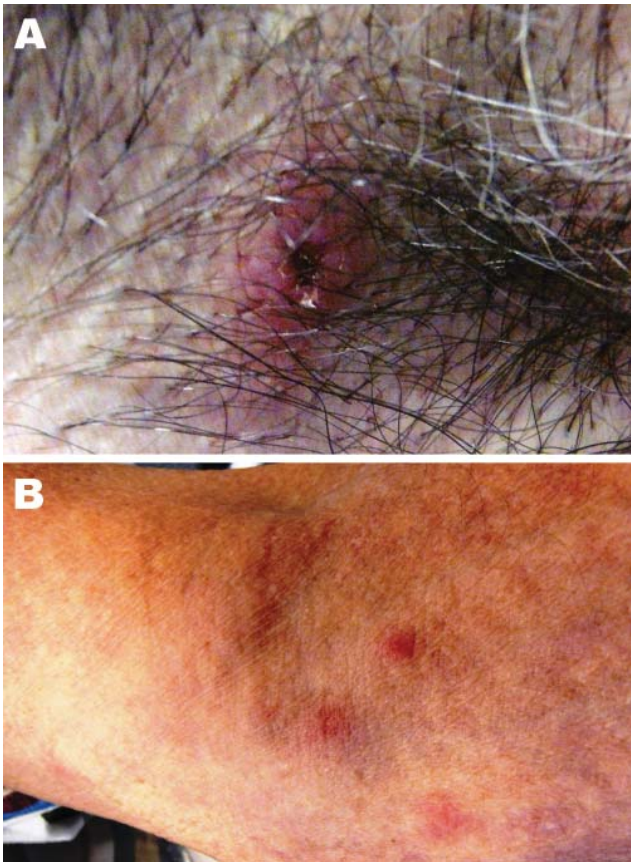

Figure. Images of lesion in the patient caused by bite from lone star tick. A) Erythematous circular lesion in right armpit at site of tick bite with induration and a necrotic center. B) Maculopapular rash involving the inferior portion of the arm. Source: Julie M. Bradley.

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The patient provided the tick, which he had preserved in alcohol. The tick was identified by using Herms's taxonomic key to *Ixodes* spp. ticks as a male lone star tick (*A. americanum*). Amplification of the mitochondrial 16S rRNA gene confirmed the tick species as *A. americanum* (417/418 bp, 99.8% homology with GenBank sequence L34313) (*6*).

Acute-phase serum was collected and stored until the convalescent-phase sample was obtained. Subsequently, seroconversion to *R. rickettsii* antigens was documented (reciprocal acute-phase titer 64, convalescent-phase titer 512 after 4 weeks). The patient did not seroconvert to *Ehrlichia* sp. antigens.

Rickettsia and *Ehrlichia* spp. PCRs were performed immediately. A previously described *Rickettsia* sp. PCR (primers 107F and 299R) was used to target a 209–212-bp fragment of a variable region of the outer membrane protein (*omp*) *A* gene, with species identification determined by DNA sequencing (*7*). Amplicons were obtained from the patient's blood and from the tick. After direct sequencing and comparative alignment with GenBank sequences, identical *R. rickettsii ompA* sequences were obtained from the patient's blood and the tick (Table). PCR using 17-kDa primers (17k-5s and 17k-3) were used to further support infection with *R. rickettsii* (*8*). An amplicon with 100% homology (502/502 bp) with *R. rickettsii* 17-kDa surface antigen precursor (*omp*) gene, GenBank sequence AY281069, was obtained from the tick, and a 17-kDa amplicon was obtained from the patient's blood, but the DNA concentration was too low for sequence analysis. When the *ompA* and 17-kDa sequences from the tick and the *ompA* sequences from the patient's blood were compared with those from *R. amblyommii*, *R. parkeri*, and *R. rickettsii*, sequences matched *R. rickettsii* (Table). A PCR result for *Ehrlichia* spp. DNA was negative for samples taken from the tick and the patient.

According to CDC criteria, RMSF developed in this patient after he was bitten by an *A. americanum* tick. Evidence to support a diagnosis of RMSF included 1) amplification of *R. rickettsii* DNA from the patient's blood sample at the onset of illness; 2) development of a prototypical rickettsial illness, including fever, rash, and thrombocytopenia 8 days after the tick was removed; 3) amplification of *R. rickettsii* from the alcohol-stored tick removed by the patient; 4) documentation of seroconversion to *R. rickettsii* antigens; and 5) a rapid and appropriate clinical response after treatment with doxycycline. The patient was not aware of any other recently attached ticks. Therefore, infection by an *A. americanum* tick caused by preexisting rickettsemia in the patient seems unlikely.

Conclusions

On the basis of historical (*4*) and recent PCR data, transmission of *R. rickettsii* by *A. americanum* ticks is most likely an infrequent event in North America (*9*). However, in Central and South America, *A. cajennense* and *A. imitator* are suspected vectors of *R. rickettsii* (*5*). For clinical and surveillance purposes, it is essential to recognize that at least 3 tick genera, *Dermacentor*, *Rhipicephalus*, and *Amblyomma*, may be capable of transmitting RMSF in the eastern and south-central United States.

During the past decade, CDC has reported a progressive rise in cases of RMSF, particularly in the eastern United States (www.cdc.gov/ticks/diseases/rocky_mountain_spotted_fever/statistics.html). During the same period, there has been a concurrent expansion in the geographic range of *A. americanum* ticks in conjunction with reports suggesting an increased frequency of attachment of this tick species to animals and humans (*10*,*11*). Although *R. ambylommii* is frequently amplified from *A. americanum* ticks, no current evidence supports a pathogenic role for this *Rickettsia* sp. in animals or humans (*11*). In contrast, *A. americanum* ticks can transmit *R. parkeri*, which is a recently documented human pathogen, most often transmitted by the Gulf Coast tick (*A. maculatum*) (*12*,*13*). Eschars have occurred frequently in the limited number of *R. parkeri* cases published, whereas eschars, such as the one seen in this case, are uncommonly described in association with RMSF (*14*). Using the 180-bp *ompA* gene variable region we sequenced in this study, we differentiated *R. rickettsii* from *R. parkeri* and *R. ambylommii* by 9 and 21 bp, respectively. Substantial efforts to amplify *R. parkeri* by using multiple primer sets (data not shown) from the tick and the patient's blood were not successful.

Taken in the context of other recent studies, this report supports the hypothesis that lone star ticks can transmit

Table. Sequence similarities for the *ompA* and *17-kDa* genes from *Rickettsia* spp. amplified and sequenced from the patient's blood and from the *Amblyomma americanum* tick, North Carolina, 2010*

	ompA			17-kDa				
Characteristic	*R. rickettsii*	*R. parkeri*	*R. amblyommii*	*R. rickettsii*	*R. parkeri*	*R. parkeri*	*R. amblyommii*	*R. amblyommii*
GenBank no.	DQ002504	U43802	AY062007	AY281069	EF102237	U17008	AY375162	U11013
Tick sequence similarities, bp	180/180	171/180	159/180	502/502	496/497	486/489	480/490	479/489
Patient sequence similarities, bp	176/176	159/180	NA	NA	NA	NA	NA	NA

**ompA*, outer membrane protein A gene; NA, amplicon not obtained from patient's blood sample for sequence comparison.

R. ambylommii, *R. parkeri*, and *R. rickettsii* infections to persons in the United States (*12*,*13*,*15*). An increasing diversity of competent tick vectors in conjunction with recent identification of novel *Rickettsia* spp. may be contributing to the increase in seroepidemiologic surveillance trends reported for RMSF. Because extensive serologic cross-reactivity exists among rickettsial species, defining the infecting species requires organism isolation in a biosafety level III laboratory or PCR amplification and DNA sequencing, as was used in this study.

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Tick-Borne Encephalitis Virus, Kyrgyzstan

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Tick-borne encephalitis virus (TBEV) is an emerging pathogen in Europe and Asia. We investigated TBEV in Kyrgyzstan by collecting small mammals and ticks from diverse localities and analyzing them for evidence of TBEV infection. We found TBEV circulating in Kyrgyzstan much farther south and at higher altitudes than previously reported.

Tick-borne encephalitis virus (TBEV) is a flavivirus in the family *Flaviviridae*. The TBEV positive-sense RNA genome is translated as a polyprotein and subsequently cleaved into 3 structural and 7 nonstructural (NS) proteins (*1*). TBEV has 3 subtypes— European, Siberian, and Far-Eastern—each of which has its own ecology, clinical presentation, and geographic distribution (*2*). The vectors are *Ixodes ricinus* ticks for the European subtype and *I. persulcatus* ticks for the other 2 subtypes. TBEV circulates through a complex cycle involving small mammals, ticks, and large mammals (*3*); it can also be transmitted through consumption of unpasteurized milk and milk products (*4*).

Our unpublished data and that of others suggest that TBEV circulates in Kazakhstan. However, we have found no reports (in English) since 1978 of TBEV infection in the neighboring Kyrgyz Republic (Kyrgyzstan). Kyrgyzstan has extensive alpine and subalpine habitats (94% of Kyrgyzstan is ≥1,000 m above sea level) (*5*); the Tien Shan mountain range dominates and physiographically links Kyrgyzstan to the Himalayas and western People's Republic of China. We conducted fieldwork in Kyrgyzstan during June–July 2007 and July–August 2009 to establish a baseline of risk for zoonotic diseases, including TBEV.

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The Study

During the 2007 and 2009 study periods, we collected 369 rodents and insectivores and 222 ixodid and 128 argasid ticks from 6 localities in Kyrgyzstan (Figure 1; Table 1) in accordance with animal subject review boards of Texas Tech University and the State University of New York at Buffalo. We analyzed 302 rodents and insectivores for immunoglobulin (Ig) G and IgM to TBEV by using recombinant antigen of domain III from the envelope (E) protein of Kumlinge and Powassan viruses (*6*). This assay is specific for the tick-borne flavivirus group and lacks cross-reactivity that occurs with other assays (*7*). We found that serologically positive (IgG and IgM) mammals were clustered at Ala-Archa National Nature Park, ≈40 km south of Bishkek, the capital of Kyrgyzstan, at elevations ranging from 1,891 to 2,472 m. Using mitochondrial DNA analysis, we also found clusters of seropositive Himalayan field mice, *Apodemus pallipes*.

To further evaluate the prevalence of TBEV, we used reverse transcription–PCR (RT-PCR) to examine viral genomic sequences in tissue samples collected from rodents, insectivores, and ticks. We used 3 separate PCR protocols. Table 2 shows primer sequences. Real-time and conventional RT-PCRs were used; however, conventional RT-PCR was preferred because it allowed sequencing of viral genomes. Thus far, we have examined sequences from the NS5 (*8*) and E (*9*) protein coding regions.

On the basis of data obtained in 2007, we focused collections in 2009 at 2 sites at Ala-Archa, 5 km apart and differing in elevation by 100 m. We found TBEV-positive ticks and IgG- and IgM-positive *A. pallipes* mice at collection sites. Sequence analyses of TBEV NS5 and E genes from *A. pallipes* mice and *I. persulcatus* ticks suggested that the TBEV circulating in Kyrgyzstan is

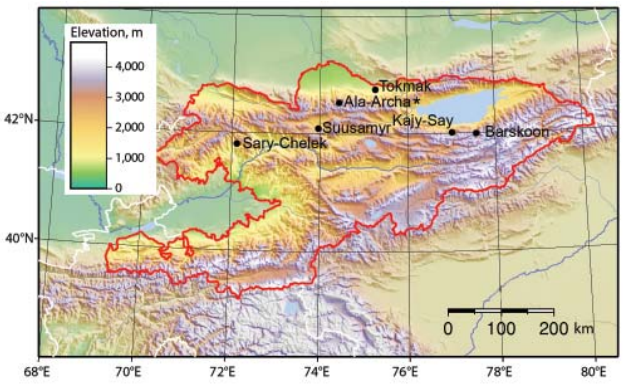

Figure 1. Animal trapping sites in Kyrgyzstan, with topographic characteristics shown. Ala-Archa (star) is the location of tick-borne encephalitis virus and a possible human case of tick-borne encephalitis.

Table 1. Overview of samples collected and tested for tick-borne encephalitis virus, 6 localities, Kyrgyzstan*

Animal species	No. collected		No. positive in Jun–Jul 2007/Jul–Aug 2009 (overall %)		
	Jun–Jul 2007	Jul–Aug 2009	RT-PCR†	IgG	IgM
Alticola argentatus	2	5	1	0	0
Apodemus pallipes‡	79	93	3	5/10 (8)	2/9 (6)
Apodemus agrarius	11	15	0	0	0/5 (19)
Crocidura sp.	11	2	0	0	0
Dryomys nitedula	11	0	0	0	0
Microtus ilaeus§	39	17	3	0/1 (2)	3/0 (5)
Microtus gregalis	0	1	0	0	0
Mus musculus	3	10	0	1/1 (15)	0/2 (15)
Myodes centralis	1	31	0	0/2 (6)	0/2 (6)
Rattus turkestanicus	26	0	0	1/0 (4)	1/0 (4)
Rattus norvegicus	1	0	0	0	0
Cricetulus sp.	0	4	0	0	0/1 (25)
Meriones sp.	0	6	0	0	0
Total	184	185	7	7/14	6/19

*Analysis of reverse transcription–PCR (RT-PCR) and ELISA. RT-PCR data represent only 2007 samples. Ig, immunoglobulin.
†5 of 7 positive samples were collected from Ala-Archa.
‡1 positive *A. pallipes* mouse was collected from Sary-Chelek.
§1 positive *M. ilaeus* vole was collected from Suusamyr.

of the Siberian subtype. Phylogenetic analyses of the E protein, amplified from a pool of *I. persulcatus* ticks collected at Ala-Archa, showed that the TBEV strain from Kyrgyzstan shares a clade with 2 strains (TBEV 1467 and Z6) isolated in the Novosibirsk region of Russia (Figure 2). This sequence is homologous with that of 5 other TBEV-positive tick pools and liver samples from *A. pallipes* mice from the same collection site.

While we were conducting fieldwork in 2009, a possible case of encephalitis occurred in a human. The 21-year-old man had removed an engorged tick (*I. persulcatus*) from himself after visiting Ala-Archa in June 2009. After ≈22 days, he sought care at the National Center for Infection in Bishkek for signs and symptoms consistent with viral encephalitis; he died 15 days later. We obtained 2 samples of the patient's serum (in accordance with approved human Institutional Review Board protocols from the State University of New York at Buffalo) at 22 and 37 days postexposure. The TBEV IgG titer for the 22-day sample was 200, within reference range, but the titer at 37 days was 2,000. This rising titer strongly indicates infection with TBEV.

Conclusions

Identification of the Ala-Archa National Nature Park as a focus of TBEV transmission is noteworthy because of its proximity to the capital. This TBEV focus is unlikely to be transient because we found evidence of TBEV in small mammals and ticks in 2007 and in 2009. We also found serologic evidence of human infection in 2009. Our findings are relevant to public health because Ala-Archa is frequently visited by hikers and climbers from many parts of the world. In 2008, nearly 45,000 persons visited Ala-Archa.

A TBEV focus at 2,100 m on the north slope of the Tien Shan mountains is relevant for several reasons. One of these is the fact that the east–west Tien Shan mountain range marks the approximate southernmost distribution of *I. persulcatus* ticks, the vectors of the Siberian and Far-Eastern strains of TBEV (*10*). Likewise, the north slope of this mountain range marks the northernmost distribution of the likely reservoir species in Kyrgyzstan, *A. pallipes* mice. Our analysis of cytochrome b DNA sequences from these mice in Kyrgyzstan supports the hypothesis that they are recent, Late Pleistocene or Holocene epoch (<15,000

Table 2. Primers used to test rodents, insectivores, and ticks for tick-borne encephalitis virus, Kyrgyzstan, 2007 and 2009*

Primer	Gene	Sequence, 5′ → 3′	Reference
FSM-1	NS5	GAGGCTGAACAACTGCACGA	(*8*)
FSM-2	NS5	GAACACGTCCATTCCTGATCT	(*8*)
FSM-1i	NS5	ACGGAACGTGACAAGGCTAG	(*8*)
FSM-2i	NS5	GCTTGTTACCATCTTTGGAG	(*8*)
TBEV913F	E	TGCACACAYYTGGAAAACAGGGA	(*9*)
TBEV1738R	E	TGGCCACTTTTCAGGTGGTACTTGGTTCC	(*9*)
RH TBE forward	E	GGCAGCATTGTGACCTGTGT	R. Hewson, unpub. data
RH TBE reverse	E	CGTGTCCTGTGGCTTTCTTTTT	R. Hewson, unpub. data
RH TBE probe	E	6FAM-AGGYGKCYTGTGAGGC-MGB NFQ	R. Hewson, unpub. data

*TBEV, tick-borne encephalitis virus; NS, nonstructural protein; E, envelope.

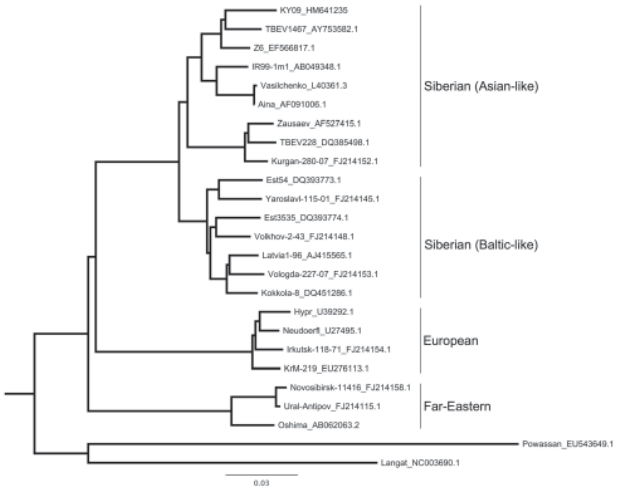

Figure 2. Maximum-likelihood phylogenetic tree of relationship between various tick-borne encephalitis virus (TBEV) strains isolated from rodents, insectivores, and ticks, Kyrgyzstan, 2007 and 2009. Tree is based on partial sequencing of the envelope protein (from Cys3 to Gly286). Strain names are followed by GenBank accession numbers. The strain from Ala-Archa (KY09_HM641235) is most closely related to strains from Novosibirsk (TBEV 1467 and Z6). This strain was isolated from an *Ixodes persulcatus* tick pool, representative of 5 other positive tick pools, and from liver samples from 2 *Apodemus pallipes* mice (sequence analysis of other samples not shown). Scale bar indicates nucleotide substitutions per site.

years), arrivals in the region. Haplotype divergence across all collecting localities is <1%. A tentative explanation for the TBEV focus on the northern slope at Ala-Archa is that this slope is the approximate geographic point of overlap for the distribution of *I. persulcatus* ticks and a suitable reservoir species, *A. pallipes* mice. Although *A. pallipes* mice have not previously been identified as a TBEV reservoir, other *Apodemus* spp. mice in Europe and Siberia are TBEV reservoirs. Laboratory studies have shown that, in contrast with other rodents, mice of the genus *Apodemus* are capable of vertical transmission (*11*) and nonviremic transmission of TBEV through ixodid ticks (*12*).

Finding TBEV-infected ticks active at these altitudes is probably not the result of climate change. Rather, we propose altitude compensation at southern latitudes as an explanation. By altitude compensation, we mean that the closer one gets to the equator, the higher the altitude that is needed for ideal transmission ecology. We suggest that TBEV transmission in Kyrgyzstan is a delicate interaction between tick larvae, tick nymphs, and reservoir rodents, analogous to the situation seen with *I. ricinus* ticks in central Europe (*13*).

Our findings provide testable hypotheses about the ecologic and physiographic factors that determine the distribution of TBEV in Kyrgyzstan. Additional understanding of these factors will aid public health responses to the zoonosis caused by this virus (*14*).

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Mr Briggs is an MD/PhD student at the State University of New York at Buffalo, School of Medicine and Biomedical Sciences. His research interests include the effects of zoonotic viruses in central Asia.

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Probable Non–Vector-borne Transmission of Zika Virus, Colorado, USA

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Clinical and serologic evidence indicate that 2 American scientists contracted Zika virus infections while working in Senegal in 2008. One of the scientists transmitted this arbovirus to his wife after his return home. Direct contact is implicated as the transmission route, most likely as a sexually transmitted infection.

Zika virus (ZIKV), a mosquito-transmitted flavivirus, has been isolated from sentinel monkeys, mosquitoes, and sick persons in Africa and Southeast Asia (*1*,*2*). Serologic surveys indicate that ZIKV infections can be relatively common among persons in southeastern Senegal and other areas of Africa, but that ZIKV-associated disease may be underreported or misdiagnosed. In 2007, a large outbreak of ZIKV infection occurred on Yap Island in the southwestern Pacific that infected ≈70% of the island's inhabitants (*3*), which highlighted this virus as an emerging pathogen. The purpose of this study was to investigate and report 3 unusual cases of arboviral disease that occurred in Colorado in 2008.

The Study

Two American scientists (patients 1 and 2) lived and worked in the village of Bandafassi in southeastern Senegal in August 2008 while performing a mosquito-sampling project in surrounding villages. Patients 1 and 2 were men (36 and 27 years of age, respectively), and both had received the yellow fever 17D vaccine before their travel to Senegal. During their project, both patients reported being bitten often by wild *Aedes* spp. mosquitoes in the evenings while they worked. Patients 1 and 2 left Bandafassi on August 21, stayed in Dakar for 2 days, and then returned to their homes in northern Colorado on August 24. Both patients became ill 6–9 days after their return.

Symptoms in patient 1 began on August 30 and consisted of swollen ankles, a maculopapular rash on his torso, and extreme fatigue and headache, but no fever was recorded. On August 31, he experienced the same symptoms and light-headedness and chills, wrist and ankle arthalgia, and symptoms of prostatitis (perineal pain and mild dysuria). However, he remained afebrile. Fatigue and rash decreased on September 1; only residual wrist arthralgia, headache, and prostatic symptoms persisted. On September 2, two aphthous ulcers appeared on his lip. On September 3, he and his wife observed signs of hematospermia (red–brown fluid in his ejaculate) that lasted until September 7. Patient 2 experienced his symptoms during August 29–September 1, which included a maculopapular rash on his torso, extreme fatigue, headache, and swelling and arthralgia in his wrists, knees, and ankles. However, symptoms of prostatitis or hematospermia did not devlop. Acute-phase blood samples were obtained from both patients on September 2.

In patient 3 (a nurse and the wife of patient 1) similar clinical symptoms developed on September 3, including malaise, chills, extreme headache, photophobia, and muscle pain that continued through September 6. She did not have detectable fever. On September 7, a maculopapular rash developed on her torso (Figure) that expanded to her neck and thighs on the following day, and an aphthous ulcer developed on her inside lip. On September 8, arthralgia in her wrists and thumbs and conjunctivitis developed. Her acute symptoms waned over the next several days. Patient 3 had an acute-phase blood sample drawn on September 8. On September 11, she visited her primary care physician, who performed a complete blood count test and studies of hepatic function; all results were within reference ranges. Patient 2 reported wrist arthralgia for 1 month after his acute illness, and patients 1 and 3 have had recurring wrist or thumb joint arthralgia since their acute illness. Convalescent-phase blood samples were drawn on September 22 from patients 1 and 2 and on September 26 from patient 3.

Acute-phase and convalescent-phase paired serum specimens from the 3 patients were tested independently by several different laboratories. Results of virus isolation were negative for all samples when tested in Vero and *Aedes albopictus* mosquito (C6/36) cell cultures and by intracerebral inoculation of acute-phase serum of patient 3 into suckling mice. Likewise, reverse transcription–PCRs with 16 different sets of arbovirus-specific primers did not detect arboviral RNA in any of the samples. Serologic analyses (online Appendix Table, www.cdc.gov/EID/

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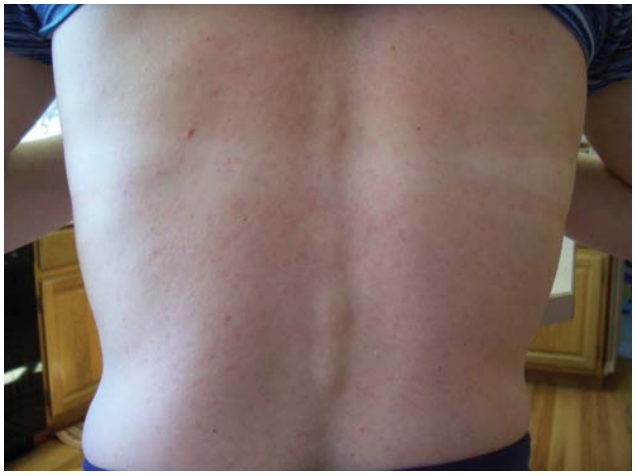

Figure. Maculopapular rash on patient 3 infected with Zika virus, Colorado, USA.

content/17/5/880-appT.htm) of samples from patients 1 and 2 showed matching results. Hemagglutination inhibition antibody titers and virus neutralizing titers were highly elevated above background levels for ZIKV and yellow fever virus (YFV) compared with other viruses tested. These titers most often increased in the time between obtaining acute-phase and convalescent-phase serum samples. Complement fixation tests against ZIKV and YFV antigens confirmed these interpretations. Hemagglutination inhibition, complement fixation, and virus neutralizing titers against ZIKV alone developed only in the convalescent-phase sample of patient 3.

Conclusions

Evidence suggests that patients 1 and 2 were infected with ZIKV, probably in southeastern Senegal, by bites from infected mosquitoes. The village of Bandafassi is located in a disease-endemic area where ZIKV has been isolated from humans, nonhuman primates, and mosquitoes (*4*,*5*). Serologic results suggest an anamnestic response to ZIKV infection, likely stemming from their vaccination with YFV. The time between infection and the onset of clinical manifestations can be inferred to be ≥9 days, given the patients' travel history. Their clinical symptoms are consistent with reported symptoms of ZIKV-associated disease (*3*,*6*–*9*). Exceptions are aphthous ulcers in patient 1 (also reported by patient 3), prostatitis, and hematospermia. Whether these exceptions are typical but unreported symptoms or clinical anomalies is not clear.

Results also support ZIKV transmission from patient 1 to patient 3. Patient 3 had never traveled to Africa or Asia and had not left the United States since 2007. ZIKV has never been reported in the Western Hemisphere. Circumstantial evidence suggests direct person-to-person, possibly sexual, transmission of the virus. Temperatures and mosquito fauna on the northern Front Range in Colorado when transmission occurred do not match known mosquito transmission dynamics of ZIKV by tropical *Aedes* species. Patient 3 had ZIKV disease 9 days after the return of her husband from Senegal. However, the extrinsic incubation period of ZIKV in *Ae. aegypti* mosquitoes was >15 days at 22°C–26°C (*10*). Area temperatures during the week of return of patient 1 fluctuated between 10°C and 31°C, only *Ae. vexans* mosquitoes of the subgenus *Aedimorphus* are commonly captured on the northern Colorado Front Range, and known tropical ZIKV vectors are mostly from the subgenus *Stegomyia* (*4*). Mosquito sampling around the home of patients 1 and 3 at the time yielded only 7 *Ae. vexans* mosquitoes and 11 other mosquitoes of the *Culex* and *Culiseta* genera.

Furthermore, patients 1 and 3 reported having vaginal sexual intercourse in the days after patient 1 returned home but before the onset of his clinical illness. It is reasonable to suspect that infected semen may have passed from patient 1 to patient 3 during coitus. Another possibility is that direct contact and exchange of other bodily fluids, such as saliva, could have resulted in ZIKV transmission, but illness did not develop in the 4 children of patients 1 and 3 during this time.

To the best of our knowledge, human sexual transmission of an arbovirus has not been documented. However, Japanese encephalitis virus was discharged into the semen of experimentally infected boars and could infect female pigs by artificial insemination (*11*). Furthermore, West Nile virus RNA and St. Louis encephalitis virus antigen have been detected in urine of humans (*12*,*13*), and viruria has occurred in hamsters infected with West Nile virus (*14*) and Modoc virus (*15*). If sexual transmission could be verified in subsequent studies, this would have major implications toward the epidemiology of ZIKV and possibly other arthropod-borne flaviviruses.

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Tick-Borne Relapsing Fever Borreliosis, Rural Senegal

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Detecting spirochetes remains challenging in cases of African tick-borne relapsing fever. Using real-time PCR specific for the 16S rRNA *Borrelia* gene, we found 27 (13%) of 206 samples from febrile patients in rural Senegal to be positive, whereas thick blood smear examinations conducted at dispensaries identified only 4 (2%) as positive.

Tick-borne relapsing fever (TBRF), caused by several species of *Borrelia* spirochetes, is transmitted to humans through the bites of soft ticks of the genus *Ornithodoros* (through infected saliva or entry of infected coxal fluid at the bite site) (*1*). Wild rodents and insectivores are common reservoir hosts. TBRF-endemic foci persist around the world, where each *Borrelia* species causing relapsing fever appears to be specific to its tick vector. TBRF is responsible for recurring fever associated with spirochetemia. In recent years, the extent of relapsing fever caused by infection with *B. crocidurae*, transmitted by *O. sonrai* ticks and its effects on public health have only just begun to emerge. In Senegal, Mali, Mauritania, and the Gambia, where this tick is endemic, 2%–70% of animal burrows are inhabited by this tick vector, and an average of 31% of ticks are infected by *B. crocidurae* (*2*,*3*).

In Senegal, TBRF caused by *B. crocidurae* was recently determined to be the most common bacterial infection affecting the human population (*3*). A conventional diagnosis of TBRF is based on the detection of spirochetes in blood smears sampled during the acute febrile phase. However, TBRF is underdiagnosed in most disease-endemic areas, where blood smears are screened only for malaria parasites. Therefore, we used specific semiquantitative PCR to evaluate the role of TBRF as a cause of fever among malaria smear–negative patients in rural Senegal.

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The Study

During December 2008 through June 2009, we enrolled all patients with fever (axillary temperature >37.5°C) who had visited the dispensaries in Dielmo (13°43′N, 16°25′W; population 391) and Ndiop (14°33′N, 16°15′W; population 313) in Senegal (*3*). Informed consent was obtained from patients and from parents or legal guardians for children. Ethical clearance was granted by the national ethics committee of Senegal and the local ethics committee of Marseille, France.

If Giemsa-stained thick and thin blood smears were negative for malaria infection, then slides were checked again for *Borrelia* spp. A first screening was made at the dispensary and a second in Dakar by highly trained microscopists. Patients whose results were positive for *Borrelia* spp. received a 7-day regimen of oral doxycycline or erythromycin (for children <8 years of age and for pregnant women). In addition, 200 μL of whole blood was collected from each malaria-negative patient and used for DNA extraction with a QIAamp kit (QIAGEN, Hilden, Germany). Samples were washed and bound with QIAGEN columns and an adapted manual polyvinyl chloride pump (Fisher Scientific Inc., Strasbourg, France). The columns were stored at 4°C until final elution and PCRs were performed in Marseille.

Borrelia DNA was detected by using specific semiquantitative real-time PCR with primers (Bor16S3F, 5′-AGC CTT TAA AGC TTC GCT TGT AG-3′; Bor16S3R, 5′-GCC TCC CGT AGG AGT CTG G-3′), and a probe (Bor16S3P, 5′-6FAM- CCG GCC TGA GAG GGT GAA CGG-3′) that were designed for amplification of a 148-bp fragment of a 16S RNA–encoding gene. The specificities of the *Borrelia* spp. detection systems had been tested on DNA samples from 347 bacterial species, as described (*4*). All real-time PCRs were performed by using LightCycler 2.0 equipment and software (Roche Diagnostics GmbH, Mannheim, Germany). Appropriate handling and DNA extraction were controlled by qualitative PCR of the β-actin gene. Negative controls (sterile water and DNA from a sterile biopsy specimen) and positive controls (*B. crocidurae* DNA) were included for each test. All positive samples with a cycle threshold level of log-based fluorescence <36 (≈10–20 copies of spacer) were used to amplify the 148-bp gene fragments, which were subsequently sequenced, by PCR (*5*). Monthly variations were analyzed by making autocorrelation correlograms with PASW software version 17 (SPSS, Chicago, IL, USA).

A total of 134 patients were included in the study, and 206 samples were obtained. Several patients were seen multiple times with fever during the study period, and 1 sample was obtained during each independent febrile event. Test results for all controls were as expected. A total of 27 samples from 25 (13%) patients (17 male) were positive

for *Borrelia* spp. by real-time PCR, including 26 (15.0%) of 172 from Dielmo and 1 (0.3%) of 34 from Ndiop. Most *Borrelia* spp.–positive patients (13; 56%) were <10 years of age, including 6 who were <5 years of age. A total of 8 (32%) patients were 5–10 years of age, and 3 were >20 years of age. Incidence rates in Dielmo were from 0.26 in November 2008, 0.51 in December 2008 and January 2009, 0.77 in March and May 2009, and 1.79 in June and July 2009.

Among the 27 samples positive by real-time PCR, only 4 (15%) had been identified as positive by thick smears at the dispensary, and only 15 (56%) had been identified as positive by highly trained microscopists who conducted a second thick-smear screening in Dakar. After PCR results were known, thick smears were examined again, and 3 more were found to be positive.

Two patients, 1 man and 1 woman, had *Borellia* spp.–positive results by real-time PCR and by thick smears when they were seen 2 times within 11 and 49 days, respectively. Among the group of 179 PCR-negative samples, no *Borrelia* spp. had been observed on thick smears. All DNA sequences obtained from these samples were identical and showed 100% identity with *B. crocidurae, B. duttonii*, *B. hispanica*, and *B. parkeri*.

Conclusions

Our specific semiquantitative PCR results demonstrated that the sensitivity of thick blood smear analysis was dramatically low (15%) when performed in standard conditions in a dispensary and remained low (56%) even when performed by trained microscopists. Similar results have recently been shown for patients infected with *B. hispanica* in Morocco (*6*) or TBRF patients in Tanzania and Togo (*7*,*8*). In the study reported here, a limited quantity of DNA was available because other causes of fever were also screened (J.F. Trape, unpub. data). Therefore, we did not amplify and sequence a larger portion of the 16S RNA gene to definitively distinguish the *B. crocidurae* endemic to Senegal from other TBRF-causing borreliae.

This study highlights the endemicity of TBRF in this rural area of western Africa, where villagers are settled agricultural workers (Figure) (*3*). Rodent and insectivore burrows are found in almost all households; burrow openings were located inside the bedrooms of traditional huts built with mud and of houses with cement floors and walls. Transmission is mainly nocturnal. The bites are painless, and tick blood meals last from a few minutes to half an hour (*9*).

B. crocidurae accounts for high fever, frequent neurologic complications (*10*), and up to 9 recurrences over several months, but the mortality rates and early delivery by pregnant women caused by *B. crocidurae* seem to be lower than those caused by *B. duttonii* (*3*). In addition to relapses, repeat infections in the same person are common (*8*), as found in 2 patients reported here. Molecular methods showed the proportion of TBRF in febrile patients to be as high as 15% in Dielmo and incidence rates to be up to 1.79% per month in June and July of 2009. This finding confirms the increased incidence of TBRF that was noted between 1996 and 2002 (*11*).

Figure. A) Dielmo village in Senegal. B) Health and clinical research station in Dielmo, where a longitudinal prospective study for long-term investigation of host–parasite associations has been conducted since 1990.

Ideally, patients living in Senegal with unexplained fever should be tested for TBRF by molecular methods. However, this technique is more readily available to travelers returning from this country (*12*) than to its citizenry.

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Novel Bluetongue Virus Serotype from Kuwait

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Sheep and goats sampled in Kuwait during February 2010 were seropositive for bluetongue virus (BTV). BTV isolate KUW2010/02, from 1 of only 2 sheep that also tested positive for BTV by real-time reverse transcription–PCR, caused mild clinical signs in sheep. Nucleotide sequencing identified KUW2010/02 as a novel BTV serotype.

Bluetongue virus (BTV) infects ruminants, camelids, and occasionally large carnivores. Clinical signs of bluetongue disease (BT) are usually more severe in sheep or white-tailed deer, particularly in populations previously unexposed to the virus; cattle and goats are often asymptomatic (*1*). Initial diagnosis of BT based on clinical signs can be confirmed by virus isolation and characterization or identification of viral RNA by reverse transcription PCR.

BTV particles contain 3 concentric protein layers surrounding 10 linear double-stranded RNA genome segments, identified as segment-1 to segment-10 (Seg-1 to Seg-10) in order of decreasing size (from 3,954 bp to 822 bp) (*2*). Twenty-five BTV serotypes have been identified on the basis of the specificity of reactions with neutralizing antibodies generated by their mammalian hosts (*3*). Consequently, BTV outer capsid proteins, particularly viral protein (VP) 2 (encoded by Seg-2), show sequence variations that determine virus serotype (*4*). Other BTV proteins, including subcore shell protein VP3(T2) encoded by Seg-3, are more highly conserved (*2*). Phylogenetic comparisons of Seg-3 sequences have been used to identify different BTV topotypes and distinguish different *Orbivirus* species (*4*).

BTV has been reported in several Middle Eastern countries (Egypt, Jordan, Syria, Turkey, Cyprus, and Iraq) since 1951 (*5*). In 2008, Egypt reported the absence of BT, and Egypt is the only country in the region to have prohibited BTV vaccination (*5*). Iran reported outbreaks of BT in 2008, and Saudi Arabia reported infection without clinical signs, although the serotype(s) were not identified (*5*). Multiple serotypes were detected in Israel during 2008 (*5*) and Oman in 2009 (S. Maan et al., unpub. data). We report characterization of a novel BTV serotype identified in Kuwait in 2010.

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The Study

During February 2010, sheep and goats in the Abdali region of Kuwait, close to the Iraq border, showed the following clinical signs consistent with BT: lameness, coughing, mouth lesions, stillbirth, congenital abnormalities, pneumonia, enteritis, and hepatitis. Forty-six of 48 serum samples were positive for BTV-specific antibodies by competitive ELISA (Investcare-Vet, London, UK) at the Veterinary Diagnostic Laboratory and Animal Research Center in Kuwait, or by double antigen-recognition ELISA (ID Vet, Montpellier, France) at the Institute for Animal Health in the United Kingdom.

Twenty-six EDTA-treated blood samples, 4 spleens, and 1 liver sent for analysis for BTV to the World Organisation for Animal Health reference laboratory at the Institute for Animal Health (Woking, UK) all gave negative results by real-time reverse transcription–PCR (rRT-PCR) targeting either BTV Seg-1 (*6*) or Seg-1 and Seg-5 (*7*). However, 2 blood samples (from animals 364 and 374) were positive in assays selective for Seg-10 (*8*), with cycle threshold (C_t) values of 35 and 28, respectively. Previous attempts to isolate BTV from blood samples with C_t values $\geq$32 were usually unsuccessful, and no further work was done with animal 364. Washed blood from animal 374 (reference collection sample KUW2010/01) (*9*) was injected into embryonated chicken eggs. Although no hemorrhages were detected, the virus was passaged twice in BHK-21 cells (isolate KUW2010/02), producing atypical cytopathic effects.

KUW2010/02 RNA analyzed by agarose gel electrophoresis generated a genome segment migration pattern (electropherotype) typical of BTV (Figure 1). Although KUW2010/02 was negative by BTV-specific rRT-PCR selective for Seg-1 (*6*), it had C_t values of 16.8 in Seg-10–specific assays (*8*). Identification of KUW2010/02 as BTV was confirmed by indirect antigen-sandwich ELISA selective for core protein VP7(T13) (*10*) with optical density at 490 nm values >0.15 (equivalent BTV titer of 6.75 $\log_{10}$ 50% tissue culture infective dose/mL).

RNA from KUW2010/01 and KUW2010/02, extracted by using TRIzol (Invitrogen, Carlsbad, CA, USA) (*11*), gave uniformly negative results by type-specific rRT-PCRs targeting Seg-2 of BTV serotypes

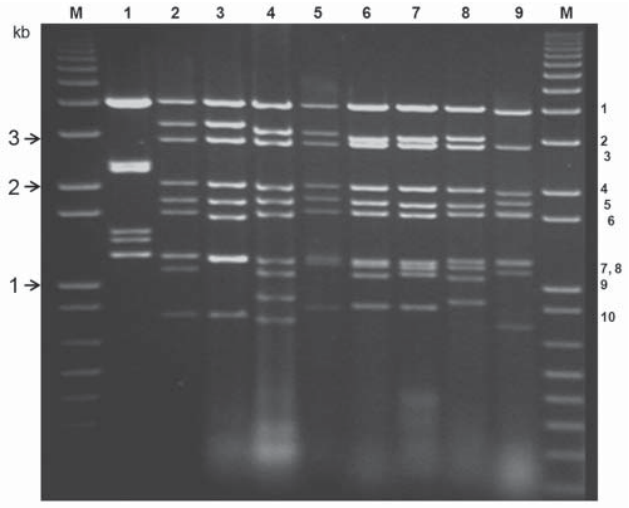

Figure 1. Electrophoretic analysis of genomic double-stranded RNAs from the *Orbivirus* species and mammalian orthoreoviruses. Bluetongue virus double stranded RNA preparations were analyzed by electrophoresis in a 1% agarose gel containing 0.5 μg/mL ethidium bromide and visualized by exposure to ultraviolet light. Genome segments are numbered, in order of decreasing molecular weight. DNA markers were run (lanes M) to enable estimation of molecular weights. Lane 1, orthoreovirus (MOR2004/01); 2, equine encephalosis virus (EEV-1/RSA1976/03); 3, African horse sickness virus (AHSV-1/RSArrah/01); 4, Palyam virus (PALV-SUD1982/03); 5, epizootic hemorrhagic disease virus (EHDV-4/ NIG1968/01); 6, bluetongue virus (BTV-15/RSArrrr/15); 7, bluetongue virus (BTV-26/KUW2010/02); 8, Tilligerry virus (TILV-AUS1978/03); 9, Chobar Gorge virus (CGV).

1–25 (kits supplied by Laboratoire Service International, Lissieu, France). However, full-length cDNA copies of Seg-2 (2,929 bp) and Seg-3 (2,773 bp) from KUW2010/02 were synthesized and then sequenced as described (*11*), showing conserved terminal regions typical of BTV (5′-GUUAAA.....ACUUAC-3′) (*2*). BLAST (www.ncbi.nlm.nih.gov/BLAST) analysis of KUW2010/02 Seg-2 and Seg-3 sequences (GenBank accession nos. HM590642 and HM590643) showed highest identity with equivalent genome segments of other BTVs, although for Seg-2 the search algorithm was changed from megablast (highly similar sequences) to blastn (somewhat similar sequences).

Phylogenetic analysis of KUW2010/02 Seg-3/VP3(T2), conducted by using neighbor-joining methods and p-distance matrices (*12*), showed nucleotide/amino acid identity levels of 73.7%/87.6% to 76.6% /88.9% with other BTVs. Seg-3 of none of the previously characterized BTVs showed close relationships to KUW2010/02; BTV-1/GRE2001/05 and BTV-25/TOV were most closely related (GenBank accession nos. DQ186822 and GQ982523, respectively), which indicates that KUW2010/02 represents a distinct geographic cluster or topotype (*4*).

Seg-2/VP2 of KUW2010/02 showed nucleotide/amino acid identity levels of 42.8%/28.3% to 63.9%/61.5% with previously recognized BTV serotypes and was most closely related to BTV-25 (nucleotype K, Figure 2). Reference strains of BTV-10 and BTV-17 from the United States (nucleotype A) were the next most closely related, with 61.8%/58.1% and 62%/57.7% nt/aa identity, respectively. This places KUW2010/02 as a distinct virus type within a novel 12th Seg-2 nucleotype L (*4*; Figure 2). ClustalX (www.clustal.org) alignments and neighbor-joining trees also confirmed the identity of KUW2010/02 as a BTV. Virus neutralization tests of KUW2010/02 (*13*) that used reference guinea pig immune serum against BTV types 1–24 and antiserum from goats previously infected with BTV-25 showed no reduction in KUW2010/02 infectivity (data not shown), demonstrating that it does not belong to BTV serotypes 1–25.

Conclusions

Most serum samples tested from sheep and goats in Kuwait showing clinical signs of disease were seropositive for BTV-specific antibodies. However, BTV RNA was detected in only 2 sheep serum samples (animals 364 and 374), suggesting that the clinical signs were not caused by ongoing BTV infection. All samples were also tested for peste des petits ruminants virus by rRT-PCR (*14*), but results were uniformly negative.

BTV RNA was detected by using a BTV Seg-10–specific rRT-PCR (*8*) previously used to detect BTV-25 in Switzerland (*15*). However, BTV Seg-1– or Seg-1– and Seg-5–specific assays (*6*,*7*) failed to detect KUW2010/02, identifying it is an unusual or atypical BTV strain. Alignment of the Seg-1–specific and Seg-5–specific primers and probes with KUW2010/02 sequences identified numerous mismatches that would prevent detection of the viral RNA (data not shown). However, the probe and primers designed by Orrù et al. (*8*) showed a perfect match with Seg-10 of KUW2010/02, demonstrating the need for appropriate rRT-PCR protocols to detect this virus. Agarose gel electrophoresis analysis of KUW2010/02 genome segments generated a migration pattern typical of BTV (Figure 1). Levels of nucleotide/amino acid identity of Seg-3 (up to 76.6%/89%) of KUW2010/02 with other BTV isolates also identified it as BTV.

At peak viremia, a previously unexposed sheep infected with a virulent BTV strain could be expected to show C_t values ≈20. The C_t of 28 obtained with blood of animal 374 (KUW2010/01) indicates a low viremia, suggesting that the severe clinical signs observed were not caused by BTV. Experimental infection of previously unexposed sheep with KUW2010/02 also caused only mild clinical signs (data not shown).

Different isolates of a single BTV serotype show >68.4%/72.6% nt/aa identity in Seg-2/VP2, with 40.5%/22.1% to 71.5%/77.8% identity between different sero-

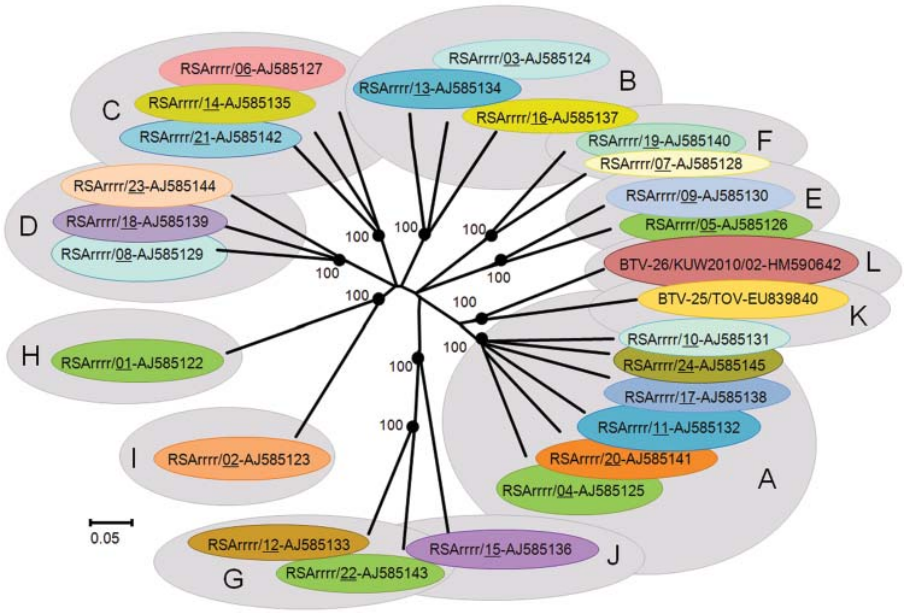

Figure 2. Neighbor-joining tree showing relationships between genome segment-2 (Seg-2) from KUW2010/02 with the 25 reference strains of different bluetongue virus (BTV) serotypes. The tree was constructed by using distance matrices, generated by using the p-distance determination algorithm in MEGA 4.1 (500 bootstrap replicates) (*12*). The 10 evolutionary branching points are indicated by black dots on the tree (along with their bootstrap values), which correlate with the 11 Seg-2 nucleotypes designated A–L. BTV-26 (KUW2010/02),forms a new 12th Seg-2 nucleotype (L). Members of the same Seg-2 nucleotype are characterized by 66.9% identity in their Seg-2 nucleotide sequences; members of different nucleotypes show 61.4% identity in Seg-2 (*4*). The scale bar indicates the number of substitutions per site. The tree based on the amino acid sequences of viral protein 2 showed very similar topology. GenBank accession numbers of Seg-2 used for comparative analyses: AJ585122–AJ585145, EU839840.

types (*4*). KUW2010/02 showed only 42.8%/28.3% to 63.9%/61.5% identity in Seg-2/VP2 with recognized BTV serotypes, consistent with membership of a novel 26th BTV type and Seg-2 nucleotype (L) (*4*). These conclusions were supported by virus neutralization test results. The sequence data presented here will help support development of new diagnostic tools (RT-PCR–based typing assays) to determine the incidence and distribution of this novel serotype, as well as natural reservoir(s) and insect vectors.

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Spotted Fever Group Rickettsiae in Ticks, Germany

Cornelia Silaghi, Dietmar Hamel, Claudia Thiel, Kurt Pfister, and Martin Pfeffer

To explore increased risk for human *Rickettsia* spp. infection in Germany, we investigated recreational areas and renatured brown coal surface-mining sites (also used for recreation) for the presence of spotted fever group rickettsiae in ticks. *R. raoultii* (56.7%), *R. slovaca* (13.3%), and *R. helvetica* (>13.4%) were detected in the respective tick species.

Rickettsia species of the spotted fever group are causing emerging infectious diseases (*1*). Since 1977, *Rickettsia slovaca*, found in *Dermacentor marginatus* ticks, was the only known *Rickettsia* sp. in Germany until 2002, when the following were identified: *R. monacensis* and *R. helvetica* in *Ixodes ricinus* ticks, *Rickettsia* sp. RpA4 (now *R. raoultii*) in *D. reticulatus* ticks, *R. felis* in *Ctenocephalides felis* cat fleas, and *R. massiliae* in *I. ricinus* ticks (*1*,*2*). All of these species cause tick-borne rickettsioses in humans, including tick-borne lymphadenopathy (TIBOLA) (*3*–*7*). The aim of this study was to explore the interface between the vector tick and humans by investigating the presence of *Rickettsia* spp. in ticks at highly frequented recreational areas and renatured brown coal surface-mining sites that also are used for leisure.

The Study

Questing ticks were collected from vegetation by flagging in 3 regions in Germany (9 sites total) in March–September 2008 and April–October 2009 (Figure). Three sites, including renatured gravel pits and walking areas near villages and cities (A–C), were located in the federal state of Saarland. One site in southern Germany was in a natural alluvial forest north of Munich, popular for hiking and dog walking (D), and in East Germany (Saxony), 3 sites were former brown coal surface-mining areas near the city of Leipzig (E–G). Ongoing renaturation and flooding of the pit holes during past decades created a highly valuable recreational area with artificial lakes and surrounding meadows and forest (www.leipzigerneuseenland.de). Here, sampling was carried out around Lake Cospuden (436 ha, fully flooded for the past 10 years, 51.5 m deep; Figure). Further sampling sites were located on a renatured former waste disposal area (H), now a popular urban recreation area, and in an alluvial forest near a popular game park (I), both within Leipzig.

Ticks were stored either in 70% ethanol or kept at 4°C, identified to species level, and homogenized (ticks in ethanol: 2 mL Eppendorf tubes [Eppendorf, Hamburg, Germany] with one 5-mm steel bead, 80 µL phosphate-buffered saline, and in Tissue Lyser [QIAGEN, Hilden, Germany] for 5 min at 30.000U/min; live ticks: 2.0 mp PRECELLYS Kit Mk28, 320 µL minimum essential medium cell culture medium [Sigma-Aldrich, Taufkirchen, Germany], in a PRECELLYS 24 dual [Peqlab, Erlangen, Germany] for 2 × 5,000 rpm for 30 s, with a 15-s break). DNA was extracted from each tick separately (females, males, nymphs) or in pools of 5 individuals (nymphs only) with the QIAGEN DNA Mini Kit (QIAGEN) by using the protocol for animal tissue. Quality and quantity of extracted DNA were tested with a NANO DROP ND-1000 spectrophotometer (Peqlab).

Ticks were screened for rickettsial DNA by using a PCR amplifying part of the *gltA* gene as described (*8*). Some tick samples positive for *Rickettsia* spp. were selected to amplify and determine the sequence of the outer membrane protein–coding genes *ompA* and *ompB* (*9*). New primers were developed for the first part of the *ompA* gene: OmpA-MMX1-for 5′-ACAAGCTGGAGGAAGCCTAGC-3′; OmpA-MMX1-rev 5′-TCTCCCGCTCCTTTGAAAACTAT-3′.

Of the 3,076 ticks collected, 1,359 were identified as *D. reticulatus* ticks (542 males, 817 females, from all

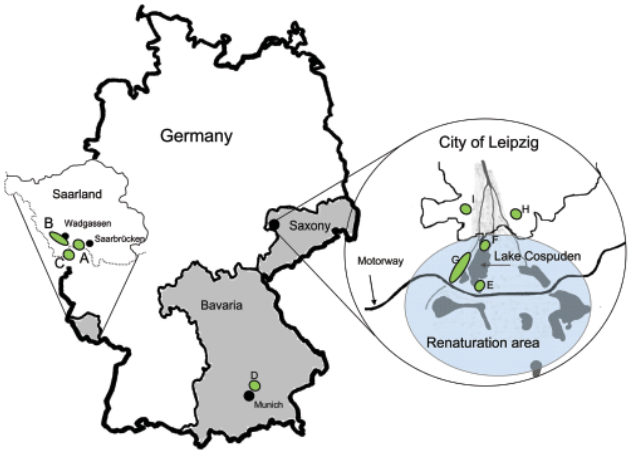

Figure. Tick sampling sites in Germany during 2008 and 2009 (green shading): Saarland, sites A–C, along the border with France (sampled March–September 2008). Bavaria, site D (sampled April and September 2009). Saxony, sites E–G, in Lake Cospuden renatured brown coal surface-mining area (blue shading); site H, renatured waste disposal site (sampled April and September/October 2009); and site I, game park (sampled in April 2009). Federal states of Saarland, Bavaria, and Saxony are shaded in gray.

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sites except site I), 15 as *D. marginatus* ticks (8 males, 7 females, only at site B), and 1,702 as *I. ricinus* ticks (714 males, 658 females, 330 nymphs, from all sites). DNA was extracted from most *D. reticulatus* ticks and almost half of the *I. ricinus* ticks (Table). In the *gltA* PCR, 849 bands of the correct size were obtained in the investigated samples (Table; 749/1,322 (56.7%) of *D. reticulatus* ticks; 2/15 (13.3 %) of *D. marginatus* ticks; 98/730 (13.4%–17.4%) of *I. ricinus* ticks (772 nymphs in pools of up to 5). The rate of infection differed by tick species, sex of the tick, and collection site; for example, the infection rate was 20.5%–76.4% in female *D. reticulatus* ticks (Table). Sequencing of 24.1% (n = 205) of the *gltA* PCR-products verified the specific amplification of rickettsial DNA in 192 cases (93.6%); sequencing for the remaining 13 amplicons could not be determined because the sequencing result was of poor quality. In 1 *I. ricinus* tick from site B, 87%–88% identity to *R. asiatica*, *R. canadensis*, and *R. helvetica* was identified, but amplification of *ompA* and *ompB* failed to further verify the species.

From each site, 1–6 positive tick samples were analyzed for the *ompA* and *ompB* genes. The *ompB* genes of *R. raoultii* (n = 27), *R. helvetica* (n = 8), and *R. slovaca* (n = 2), and *ompA* genes of *R. raoultii* (n = 25) and *R. slovaca* (n = 2) were 100% identical in the amplified part of the respective species, regardless of geographic origin. *R. raoultii* from our study showed 100% identity (*ompA*) and 99% identity (*ompB*) to *R. raoultii* strain Marne (GenBank accession nos. DQ365800 and DQ365797); for *R. helvetica* (*ompB*), 100% identity to GenBank entry AF123725; and for *R. slovaca,* 100% identity to GenBank entry AF123723 (*ompB*), and U83454 (*ompA*). *R. raoultii*, *R. slovaca*, and *R. helvetica* were found only in *D. reticulatus*, *D. marginatus*, and *I. ricinus* ticks, respectively. Sequences (*ompA* and *ompB*) from this study were deposited in GenBank (accession nos. HQ232215–HQ232278).

Conclusions

In Germany, the most common tick is *I. ricinus*; *D. reticulatus* ticks have a focal distribution, and *D. marginatus* ticks have been described on only a few occasions because the latter require warm and dry habitats (*2*,*10*,*11*). Climate change and structural landscape changes have been discussed as reasons for the creation of new tick habitats (*12*). Brown coal surface-mining sites of the former German Democratic Republic undergo extensive renaturation, thus providing new biotopes for many plant and animal species, including ticks.

In Germany, *R. slovaca* was first described in 1977 (*2*) and again recently (*11*). Even though the sample size in the present study was small, comparable prevalence rates were detected (13%). *R. raoultii* was first detected in Russia and has recently been described as a new species (*13*). The average infection rate of *R. raoultii* in this study was 56.7% and, in the renatured brown coal surface-mining sites, ≈80.1%. The latter rate is high in comparison with results of previous studies (*11*,*12*). *R. helvetica* prevalence in *I. ricinus* ticks was similar to results of other studies in Germany (*8*).

Our results confirm the presence of these rickettsial pathogens in Germany. In addition, we identified previously unknown areas where *Rickettsia* spp. are endemic. This finding is of major concern to public health: both *R. slovaca* and *R. raoultii* can cause TIBOLA, even though *R. slovaca* is considered to be more pathogenic (*7*,*14*). A case of TIBOLA caused by *R. slovaca* was identified in a human patient in an *R. slovaca*–endemic area in western Germany (*11*). The pathogenicity of *R. helvetica* has not been fully clarified, but serologic evidence shows human infections in

Table. *Rickettsia* spp. found in ticks from different sites, Germany, 2008–2009*

State (region)	Site†	No. positive/total no. (%)						
		Ixodes ricinus ticks			*Dermacentor reticulatus* ticks		*D. marginatus* ticks	
		Male	Female	Nymph‡	Male	Female	Male	Female
Saarland (West)	A	1/6 (16.6)	0/6 (0)	1–3/8 (12.5–37.5)	5/14 (35.7)	7/14 (50)	NA	NA
	B	8/26 (30.8)	6/35 (17.1)	3–7/14 (21.4–50.0)	67/128 (52.3)	71/153 (46.4)	1/8 (12.5)	1/7 (14.3)
	C	2/4 (50.0)	0/2 (0)	2–7/14 (14.3–50.0)	9/35 (25.7)	8/39 (20.5)	NA	NA
Bavaria (South)	D	11/58 (19.0)	8/42 (19.0)	6/28 (21.4)	11/40 (27.5)	30/95 (31.6)	NA	NA
Saxony (East)	E	5/56 (8.9)	10/45 (22.2)	0/6 (0)	144/190 (75.8)	236/309 (76.4)	NA	NA
	F	1/4 (25.0)	1/6 (16.6)	2–10/10 (20.0–100)	17/21 (80.1)	21/29 (72.4)	NA	NA
	G	0/2 (0)	1/6 (16.6)	1–5/13 (7.7–38.4)	30/80 (37.5)	50/111 (45.0)	NA	NA
	H	8/53 (15.1)	4/74 (5.4)	4–11/35 (11.4–31.4)	10/16 (62.5)	33/48 (68.1)	NA	NA
	I	7/104 (6.7)	5/81 (6.2)	1/34 (2.9)	NA	NA	NA	NA
Total	A–I	43/313 (13.7)	35/297 (11.7)	20–50/162 (12.3–30.1)	293/524 (55.5)	457/798 (57.1)	1/8 (12.5)	1/7 (14.3)

*NA, not available.
†For exact location of site, see Figure.
‡In the regions Saarland and Saxony, some of the nymphs were placed in pools of up to 5 specimens; range of positive ticks indicates the possible minimum and maximum number of ticks positive for *Rickettsia* spp. in a pool.

France (*3*), and DNA of *R. helvetica* was recently identified in a patient in Sweden who had meningitis (*15*).

Renaturation of industrial sites specifically provides new areas for human recreation and, simultaneously, new habitats for many plant and animal species. Previously nonexistent opportunities for intensive contact between vector ticks and humans are now available. Thus, human-made habitats may lead to increased emerging diseases, especially in tick-borne rickettsioses, because renaturation areas may form favorable biotopes for enhanced human–vector interactions.

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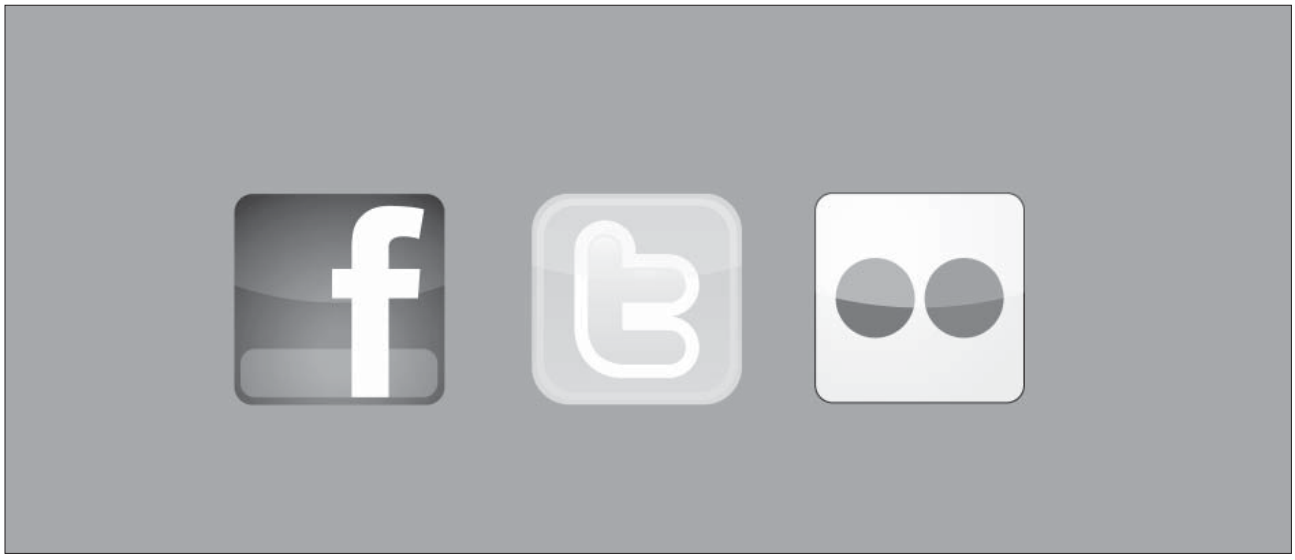

Bartonella spp. in Feral Pigs, Southeastern United States

Adam W. Beard, Ricardo G. Maggi, Suzanne Kennedy-Stoskopf, Natalie A. Cherry, Mark R. Sandfoss, Christopher S. DePerno, and Edward B. Breitschwerdt

In conjunction with efforts to assess pathogen exposure in feral pigs from the southeastern United States, we amplified *Bartonella henselae, B. koehlerae*, and *B. vinsonii* subsp. *berkhoffii* from blood samples. Feral pigs may represent a zoonotic risk for hunters or butchers and pose a potential threat to domesticated livestock.

Bartonella spp. are intravascular, gram-negative bacteria that infect a diverse array of wild and domestic animals. These bacteria appear to induce a wide range of symptoms in humans and can cause similar disease manifestations in animals (*1*,*2*). An increasing number of *Bartonella* spp. are regarded as zoonotic pathogens, which creates a public health concern for human and veterinary medicine (*3*).

Feral pigs (*Sus scrofa*), nonnative, ancestral species derived from domesticated pigs in Europe, inhabit 39 states. As their geographic distribution expands and their numbers increase, these animals are causing substantial economic and ecologic damage, which has required implementation of specific damage management programs (*4*). Hunters and butchers coming in contact with blood from feral pigs may be at risk for infection with *Bartonella* spp. (*3*). We report the molecular detection of 3 zoonotic *Bartonella* spp. in feral pigs harvested by hunters in Johnston County, North Carolina, USA.

The Study

During 2007–2009, a total of 135 EDTA-anticoagulated whole blood samples were obtained from 76 hunter-harvested juvenile and adult feral pigs (39 males). Blood samples were aspirated postmortem from the carotid artery, heart, or orbital venous sinus, resulting in ≥1 blood sample for 57 feral pigs. Specimens were stored frozen at –20°C until analysis.

DNA was extracted from EDTA anticoagulated whole blood with QIAGEN MagAttract DNA Blood Mini M48 Kits and QIAGEN BioRobot M48 Workstation (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. All 135 samples were initially screened for the *Bartonella* 16S–23S internal transcribed spacer (ITS) region by using oligonucleotides 438s (5′-GGT TTT CCG GTT TAT CCC GGA GGG C-3′) and 1100as (5′-GAA CCG ACG ACC CCC TGC TTG CAA AGC A-3′) as forward and reverse primers, respectively (*5*–*7*). Samples with positive ITS results were subsequently screened with citrate synthase, RNA polymerase B (*rpoB*), and a *B. koehlerae*–specific PCR (*6*).

For this study, a newly designed forward ITS primer (Bkoehl-1s (5′-CTT CTA AAA TAT CGC TTC TAA AAA TTG GCA TGC-3′) was used in conjunction with the 1100as reverse primer. Amplification was performed in a 25-μL final volume reaction containing 12.5 μL of Tak-Ex Premix (Fisher Scientific, Pittsburgh, PA, USA), 0.1 μL of 100 μmol/L of each forward and reverse primer (IDT DNA Technology, Coralville, IA, USA), 7.3 μL of molecular grade water, and 5 μL of DNA from each sample tested. Blood from a healthy dog was routinely used during DNA extraction and as a PCR negative (5 μL of extracted DNA) control. For positive controls, 5 μL of 0.001 pg/μL of *B. henselae* DNA (equivalent to 2.5 genome copies) was prepared by serial dilution in specific pathogen-free dog blood (*7*). No positive control was used for the *B. koehlerae* PCR. Conventional PCR was performed in an Eppendorf Mastercycler EPgradient (Eppendorf, Hamburg, Germany) under the following conditions: 1 denaturing cycle at 95°C for 2 min followed by 55 cycles at 94°C for 15 s, 68°C (*Bartonella* genus PCR) or 64°C (*B. koehlerae* PCR) for 15 s, and 72°C for 18 s. PCR was completed by an additional final cycle at 72°C for 30 sec. Products were analyzed by 2% agarose gel electrophoresis and detection by using ethidium bromide under UV light and sequenced either after purification of amplicons directly from the gel or from plasmid-clone minipreps by using QIAquick PCR purification kit or QIAGEN Miniprep Kit (QIAGEN), respectively, as described (6,*7*).

Sequence chromatograms and sequence analysis were examined by using ContigExpress software (Vector NTI Suite 10.1, Invitrogen Corp., Carlsbad, CA, USA) and BLAST version 2.0 (www.ncbi.nlm.nih.gov/Education/BLASTinfo/BLAST_algorithm.html) from GenBank. Bacteria species and strain identification was performed by using AlignX software (Vector NTI Suite 10.1, Invitrogen).

Of 76 feral pigs harvested from Johnston County, North Carolina, and tested by using the 438–1100 ITS PCR, amplicons consistent in size with a *Bartonella* spp. (400–600-bp amplicon size) were amplified and successfully sequenced from 15 (19.7%) animals. Two *B. henselae* strains, *B. koehlerae* and *B. vinsonii* subsp. *berkhoffii* genotypes I and III, were identified (Figure).

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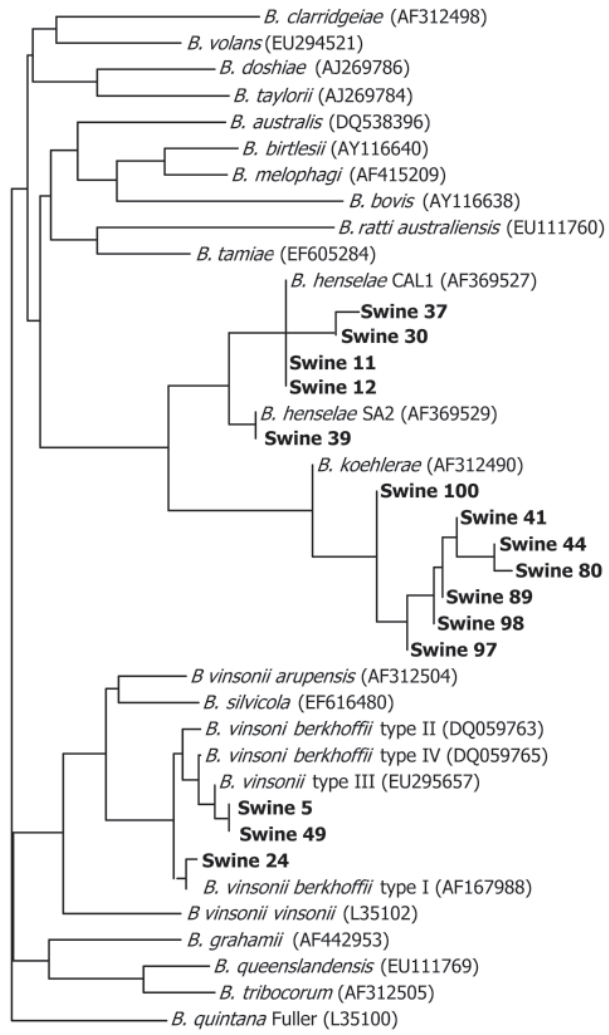

Figure. Tree pair-wise alignment of *Bartonella* DNA sequences detected in feral pig blood samples. GenBank accession numbers are in parentheses. **Boldface** indicates sequences generated in this study compared with sequences previously submitted to GenBank.

Seven *Bartonella* PCR–positive samples aligned with *B. koehlerae* with sequence similarities of 99.2%, 99.4%, 99.8%, and 100% (4 animals) to GenBank sequence AF312490. Four sequences aligned with *B. henselae* strain Cal-1 (GenBank accession no. AF369527) with 98.7%, 99.1%, 99.4% (2 animals) sequence similarities. *B. henselae* strain SA2 (San Antonio 2, GenBank accession no. AF369529) was detected in an additional animal with sequence homology of 99.8%.

Three feral pig sequences aligned with 2 genotypes of *B. vinsonii* subsp. *berkhoffii* (*8*): 2 animals with 100% homology to genotype III (GenBank accession no. DQ059764), and 1 animal with 99.6% homology with genotype I (GenBank accession no. AF167988). *B. vinsonii* subsp. *berkhoffii* genotype III (99.8% homologous to DQ059764) and *B. koehlerae* (99.1%, homologous to AF312490) sequences were amplified from the same sample. Two different primer sets amplified *B. koehlerae* DNA from 3 of 7 and 1 of 2 *B. vinsonii* subsp. *berkhoffii* genotype III–infected pigs, respectively. PCR specific for the *rpoB* gene resulted in amplification of *B. vinsonii* subsp. *berkhoffii* DNA from the only *B. henselae* SA2–infected pigs. In no instance was *B. henselae* (Cal1) amplified and sequenced by using 2 primer sets. *Mesorhizobium* sequences were obtained from most of the other *rpoB* PCR amplicons and from one 325s amplicon. Previously, we have reported nonspecific amplification of *Mesorhizobium* sequences by using other *Bartonella* spp. 16S–23S ITS primers (*5*).

Conclusions

We amplified and sequenced *B. henselae*, *B. koehlerae*, and *B. vinsonii* subsp. *berkhoffii* DNA using ≥1 primer sets from 19.7% of hunter-harvested feral pigs. The domestic cat is the primary reservoir for *B. henselae* and *B. koehlerae*, and fleas are the primary vector (*1*). Managers of the study site reported the presence of feral cats, but cat numbers and interactions with feral pigs were unknown. Although feral pigs in the southeastern United States are hosts for ticks that are potential *Bartonella* vectors (*9*,*10*), the pigs in this study were harvested during the winter so no ectoparasites were found.

Mesorhizobium, an environmental microbe, most likely introduced during sample collection under field conditions, also was amplified by using 3 primer sets. Although unlikely, ectoparasite feces or dirt containing *Bartonella* spp. could have been similarly introduced during venipuncture. For future studies in which molecular testing is anticipated, blood should be collected aseptically.

The 3 *Bartonella* spp. found in feral pigs, *B. henselae*, *B. vinsonii* subsp. *berkhoffii*, and *B. koehlerae*, are known zoonotic pathogens (*3*,*11*,*12*). Transmission of *B. alsatica*, which infects wild rabbits in Europe, has been reported in humans with endocarditis and lymphadenitis in association with butchering wild rabbits (*13*). Because hunters and butchers are exposed to large quantities of pig blood, potential exists for *Bartonella* spp. transmission through inadvertent cuts or scratches, which has occurred with other zoonotic pig pathogens, such as *Brucella suis* (*14*).

Another potential implication of these results involves the transmission of *Bartonella* spp. from feral to domesticated pigs (*15*). *Ctenocephalides felis* and *C. canis* fleas, known vectors of *B. koehlerae and B. henselae,* have been reported to infest young pigs (*10*,*12*). Measures to control ectoparasites are commonly used by large commercial pig operations, where transmission of *Bartonella* spp. is not likely to pose a production or zoonotic risk.

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Rickettsia parkeri in Gulf Coast Ticks, Southeastern Virginia, USA

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We report evidence that *Amblyomma maculatum* tick populations are well established in southeastern Virginia. We found that 43.1% of the adult Gulf Coast ticks collected in the summer of 2010 carried *Rickettsia parkeri*, suggesting that persons living in or visiting southeastern Virginia are at risk for infection with this pathogen.

Rickettsia parkeri is an obligate intracellular bacterium belonging to the spotted fever group of rickettsiae; this organism has recently been found to be pathogenic to humans (*1*). Infection with *R. parkeri* can be considered an emerging infectious disease, referred to as *R. parkeri* rickettsiosis, American Boutonneuse fever, and Tidewater spotted fever. Two confirmed cases of *R. parkeri* infections, including the index case in 2002, occurred in southeastern Virginia (*1*–*3*). Since then, 20 *R. parkeri* infections have been reported, mainly from the southern United States (*2*). In the United States, *Amblyomma maculatum* (family *Ixodidae*) ticks, commonly referred to as Gulf Coast ticks, are the only known natural vector of *R. parkeri*. *A. maculatum* ticks have been reported from 12 states: Alabama, Arkansas, Florida, Georgia, Kansas, Kentucky, Mississippi, Oklahoma, South Carolina, Tennessee, Texas (*1*,*4*,*5*), and Virginia (*6*). Sonenshine et al. reported finding individual *A. maculatum* ticks in Virginia in 1965 but concluded that populations had not become established (*7*).

We found large numbers of adult and some nymph *A. maculatum* ticks in Virginia. This population and the different life stages of the ticks indicate that they are now established in the state. Testing by real-time PCR and sequencing indicated that a high percentage of the ticks contained *R. parkeri* DNA.

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The Study

From May through September 2010, adult questing *A. maculatum* ticks were collected on flags at 3 locations in southeastern Virginia. Collection sites were selected to produce results that could be compared with those of previous surveys and to provide a comprehensive survey of southeastern Virginia (*8*). The first study site is 50 km inland and borders the Great Dismal Swamp in Chesapeake, Virginia. The second site, Back Bay National Wildlife Refuge, is <1 km from the Atlantic Ocean in Virginia Beach. The third site, in Portsmouth, borders the Elizabeth River.

The ticks were identified morphologically, and identity was confirmed as needed by molecular methods. DNA was extracted by using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol and stored at –20°C until processing.

DNA samples were tested for *R. parkeri* DNA by real-time PCR with a MiniOpticon Real-Time PCR System (Bio-Rad, Hercules, CA, USA). Testing for *R. parkeri* DNA was by amplification and detection of a fragment of the *ompB* gene by using Rpa129F and Rpa224R primers and Rpa188 as the probe (Table 1). Samples negative for *R. parkeri* DNA were tested for *Rickettsia* spp. by amplifying a 111-bp fragment of the 17-kDa antigen gene (Table 1).

Three representative *A. maculatum* samples positive for *R. parkeri* by real-time PCR were confirmed by sequencing of a 540-bp fragment of the *ompA* gene. The fragments were amplified on an iCycler (Bio-Rad) by using primers 190-FN1 and 190-RN1 (Table 1). Samples positive for *Rickettsia* spp. but negative for *R. parkeri* had their *ompB* gene amplified and sequenced by using primers RompB11F and RompB1902R (Table 1). All PCR products for sequencing were purified by using Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA), and sequencing reactions were performed by using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) as described by the manufacturer and using appropriate primers (Table 1). Sequence similarities were identified by a BLAST search (http://blast.ncbi.nlm.nih.gov).

A total of 65 adult and 6 nymph *A. maculatum* ticks were collected (adults in May–September, nymphs in April). A total of 54 adults were collected from the Chesapeake site, 8 from the Virginia Beach site, and 3 from the Portsmouth site. Of the 6 nymphs collected, 5 were found feeding on a cotton rat at the Chesapeake site in April, and 1 was collected on a flag at the Virginia Beach site in September. Of the 65 total adult ticks tested, 29 (44.6%) were found by real-time PCR to contain *Rickettsia* spp. DNA, and 28 (43.1%) of the total adults collected contained *R. parkeri* DNA. Of the 6 nymphs collected, 4 were infected with *R.*

Table 1. Sequences of primers and probes used to test for *Rickettsia* spp. DNA in *Amblyomma maculatum* ticks collected from southeastern Virginia, April–September 2010*

Name	Sequence, 5′ → 3′	Gene	Fragment	Reference
Rpa129F	CAAATGTTGCAGTTCCTCTAAATG	*ompB*	96	J. Jiang et al., unpub. data
Rpa224R	AAAACAAACCGTTAAAACTACCG	*ompB*	96	J. Jiang et al., unpub. data
Rpa188Probe	6-FAM-CGCGAAATTAATACCCTTATGAGCAGCAGTCGCG-BHQ-1	*ompB*	96	J. Jiang et al., unpub. data
R17K128F2	GGGCGGTATGAAYAAACAAG	17-kDa antigen gene	111	J. Jiang et al., unpub. data
R17K238R	CCTACACCTACTCCVACAAG	17-kDa antigen gene	111	J. Jiang et al., unpub. data
R17K202TaqP	FAM-CCGAATTGAGAACCAAGTAATGC-TAMRA	17-kDa antigen gene	111	J. Jiang et al., unpub. data
190-FN1	AAGCAATACAACAAGGTC	*ompA*	540	(*1*)
190-RN1	TGACAGTTATTATACCTC	*ompA*	540	(*1*)
RompB11F	ACCATAGTAGCMAGTTTTGCAG	*ompB*	1895	(*9*)
RompB1902R	CCGTCATTTCCAATAACTAACTC	*ompB*	1895	(*9*)

**omp*, outer membrane protein gene.

parkeri; all were from the rat at the Chesapeake site. Of the *R. parkeri*–positive samples sequenced, maximum identity was seen with *R. parkeri* sequences (GenBank accession no. FJ986616.1). The rate of *R. parkeri*–infected ticks started out high in May (83% infected) and then decreased to no infected ticks in August (Table 2).

Of the 3 *A. maculatum* ticks collected from the Portsmouth site, 1 was found by real-time PCR to be positive for *Rickettsia* spp. but negative for *R. parkeri*. Sequencing of a fragment of the *ompB* gene revealed this isolate to contain DNA with a 100% match to *Candidatus* Rickettsia andeanae isolate T163 (GenBank accession no. GU395297.1), a rickettsiae initially found in Peru (*9*).

Conclusions

The discovery of such numbers and life stages of *A. maculatum* ticks in widely dispersed locations indicates that they are now established in southeastern Virginia. Finding adult *A. maculatum* ticks at the Portsmouth site was unexpected because this is the northernmost site at which we found these ticks and is a peninsula devoid of white-tailed deer, a major host for adult ticks (*10*,*11*).

That 43.1% of adult *A. maculatum* ticks collected from southeastern Virginia contained *R. parkeri* differs from reported rates of *R. parkeri* in *A. maculatum* ticks elsewhere in the United States. For *A. maculatum* ticks from Florida and Mississippi, *R. parkeri* infectivity rate is 28% (*2*); for ticks from Florida, Kentucky, Mississippi, and South Carolina, the average rate is 11.5% (*12*). For *A. maculatum* ticks collected from Georgia, an infectivity rate of 5%–11.5% has been reported (*13*). In Arkansas, only 3 of 207 *A. maculatum* ticks contained *R. parkeri* (*14*). Despite the high percentage of *R. parkeri* in the southeastern Virginia ticks, 27 of 28 positive samples came from 1 collection site. One explanation could be that *R. parkeri* is transovarially transmitted. Currently, there is no evidence that *R. parkeri* is transmitted transovarially by *A. maculatum* ticks, although transovarial transmission of *R. parkeri* has been shown in *A. americanum* ticks in the laboratory (*15*).

We also found an *A. maculatum* tick infected with *Candidatus* Rickettsia andeanae, which has rarely been reported in the United States (*2*). Whether *Candidatus*

Table 2. Real-time PCR results for adult *Amblyomma maculatum* ticks collected from southeastern Virginia, USA, 2010

Month and collection site	Total no. ticks	No. (%) positive *for Rickettsia parkeri*
May		
Chesapeake	12	10 (83)
Virginia Beach	0	0
Portsmouth	0	0
Total	12	10 (83)
June		
Chesapeake	37	15 (40.5)
Virginia Beach	4	0
Portsmouth	0	0
Total	41	15 (36.5)
July		
Chesapeake	3	2 (66.7)
Virginia Beach	1	0
Portsmouth	3	1 (33.3)
Total	7	3 (42.9)
August		
Chesapeake	1	0
Virginia Beach	2	0
Portsmouth	0	0
Total	3	0
September		
Chesapeake	1	0
Virginia Beach	1	0
Portsmouth	0	0
Total	2	0
Total	65	28 (43.1)

Rickettisa andeanae is pathogenic to humans is unknown, although it has been suspected to cause infections in persons in Peru (9).

Further research is needed to identify the vertebrate host(s) of *R. parkeri*. This information could be useful for controlling the transmission of *R. parkeri* to and from the vector, as well as predicting where *R. parkeri* may be present. Studies relating to transovarial transmission of *R. parkeri* in *A. maculatum* ticks would also be useful for predicting the spread of infections. Because *R. parkeri* is known to cause infection in humans, the presence of this pathogen in southeastern Virginia should be a health concern to persons in this region.

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Multitarget Test for Emerging Lyme Disease and Anaplasmosis in a Serosurvey of Dogs, Maine, USA

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To determine if the range of deer ticks in Maine had expanded, we conducted a multitarget serosurvey of domestic dogs (*Canis lupus familiaris)* in 2007. An extension of exposure to *Borrelia burgdorferi* to the northern border and local transmission of *Anaplasma phagocytophilum* throughout southern areas was found.

Over the past 2 decades, the range of *Ixodes scapularis*, the deer tick, vector of Lyme disease, anaplasmosis, babesiosis, and deer tick virus infections, has expanded in northern New England. Because Lyme disease and anaplasmosis affect humans and dogs (*Canis lupus familiaris*), serosurveys of canids have proved useful for monitoring emergence of these infections. Sample selection may be confounded when dogs that are remotely exposed, vaccinated, or treated with topical acaricides are included. In recent years, however, the advent of a multitarget, in-clinic test kit (SNAP 4Dx; IDEXX Laboratories, Westbrook, ME, USA) has increased the scope and efficiency of these serosurveys. The SNAP4Dx tests for heartworm antigen and antibodies to *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Ehrlichia canis* on 3 drops of blood. Its sensitivity and specificity for antibodies against *B. burgdorferi* and *A. phagocytophilum* exceed 98% (*1*,*2*).

In Maine, deer ticks were first reported at a coastal site in 1988 and have since spread inland (*3*). Lyme disease has become a major public health problem; reported human cases reached 169 per 100,000 population in 1 mid-coastal county in 2008. Human cases of anaplasmosis and babesiosis are also being reported (*4*). In 1990, we conducted a statewide serosurvey to map *B. burgdorferi*–positive dogs and to correlate their distribution with reported human cases. Four percent of 828 samples were seropositive for *B. burgdorferi*, 89% of which were from dogs residing within 20 miles of the coast. No positivity was found among 102 dogs in the northern half of the state (*5*). Given the widespread acceptance of SNAP 4Dx tests by Maine veterinarians, we resurveyed dogs statewide in 2007 for exposure to *B. burgdorferi* and *A. phagocytophilum.* Data from questionnaires to veterinarians and dog owners enabled assessment of the influence of the use of Lyme vaccines and topical acaricides on canine serologic test results.

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The Study

From 87 veterinary clinics solicited in 2007, we selected 47 on the basis of their size and geographic distribution. Each was supplied with 15–30 SNAP 4Dx kits (contributed by IDEXX Laboratories). Veterinarians were instructed to obtain samples from all dogs routinely tested for heartworm. In northern areas, where heartworm is rarely tested for, they were asked to collect samples from dogs undergoing surgery. They recorded each dog's age, town of residence, Lyme disease vaccination status (ever or never vaccinated), and the test results. Each dog owner completed a form (99.6% response rate) to describe the dog, its function, history of unexplained lameness, travel history (town, state, visited within the past year), history of tick infestation, and use of tick control products (yes or no).

We summarized test results to town and county levels. We used Spearman rank correlation tests to examine associations between canine seropositivity, human Lyme disease cases reported to the Maine Center for Disease Control and Prevention (Augusta, ME, USA) (*4*), and the number of deer ticks submitted to our laboratory in 2007. We used *B. burgdorferi* and *A. phagocytophilum* test results and questionnaire responses to cross-tabulate responses and calculate the likelihood (odds ratios) of *B. burgdorferi* and *A. phagocytophilum* positivity as a function of risk factors by using χ^2 tests of association. We considered differences significant at $p \leq 0.05$. Analyses were conducted by using SAS version 9.2 for Windows (SAS, Cary, NC, USA).

Of 1,087 dogs tested across Maine's 16 counties, 12.7% were *B. burgdorferi*–positive and 7.1% were *A. phagocytophilum*–positive (Table 1); 1.9% were co-infected. The distribution of all dogs seropositive for either pathogen is shown by town in Figure 1. At the county level, canine *B. burgdorferi* seropositivity among unvaccinated dogs correlated positively with the number of human Lyme disease cases reported for 2007($\rho_{Spearman} = 0.84$; $p<0.0001$) and the number of deer ticks submitted to our laboratory for identification ($\rho_{Spearman} = 0.63$; $p = 0.009$). In Figure 2, which shows statewide distributions by county north to south, only unvaccinated dogs are included in *B. burgdorferi*–positive data shown. Dogs had been exposed

Table 1. Canine seroprevalence of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* and Lyme diseases vaccination status, Maine, USA, 2007

		*B. burgdorferi**		*A. phagocytophilum**		Lyme disease vaccination status	
County	No. dogs tested	No. negative	No. (%) positive	No. negative	No. (%) positive	No. reporting	No. (%) vaccinated
Aroostook	59	56	3 (5.1)	59	0	59	9 (15.3)
Piscataquis	46	44	2 (4.3)	43	3 (6.5)	44	23 (52.3)
Somerset	57	55	2 (3.5)	54	3 (5.3)	55	33 (60.0)
Penobscot	77	73	4 (5.2)	73	4 (5.2)	75	30 (40.0)
Franklin	78	73	5 (6.4)	78	0	78	38 (48.7)
Washington	38	35	3 (7.9)	37	1 (2.6)	37	6 (16.2)
Hancock	54	46	8 (14.8)	53	1 (1.9)	54	24 (44.4)
Oxford	76	66	10 (13.2)	75	1 (1.3)	76	41 (53.9)
Waldo	62	57	5 (8.1)	60	2 (3.2)	62	38 (61.3)
Kennebec	120	106	14 (11.7)	114	6 (5.0)	119	82 (68.9)
Knox	87	67	20 (23.0)	83	4 (4.6)	81	44 (54.3)
Lincoln	91	75	16 (17.6)	85	6 (6.6)	85	63 (74.1)
Androscoggin	62	53	9 (14.5)	60	2 (3.2)	62	27 (43.5)
Sagadahoc	24	22	2 (8.3)	23	1 (4.2)	24	21 (87.5)
Cumberland	91	78	13 (14.3)	72	19 (20.9)	88	62 (70.5)
York	65	42	22 (34.4)	41	24 (36.9)	59	34 (57.6)
Total	1,087	948	138 (12.7)	1,010	77 (7.1)	1,058	575 (54.3)

*Tested by using SNAP 4Dx test kit (IDEXX Laboratories, Westbrook, ME, USA).

to *A. phagocytophilum* in all but 2 northern counties. At the town level, remarkably higher levels of canine *A. phagocytophilum* seropositivity were found in southern coastal Cape Elizabeth (Cumberland County) (76.5%, n = 17) and York (York County) (58.0%, n = 19) than in towns in their immediate vicinity.

Overall, 54.3% of the dogs had been vaccinated against Lyme disease. Never-vaccinated dogs were 1.5× as likely to be seropositive for *B. burgdorferi* than were vaccinated dogs (15.3% vs. 9.9%; p = 0.008) (Table 2). Vaccine use was higher in 10 southern counties where Lyme disease has become endemic (Figure 2, panel B) than in the 6

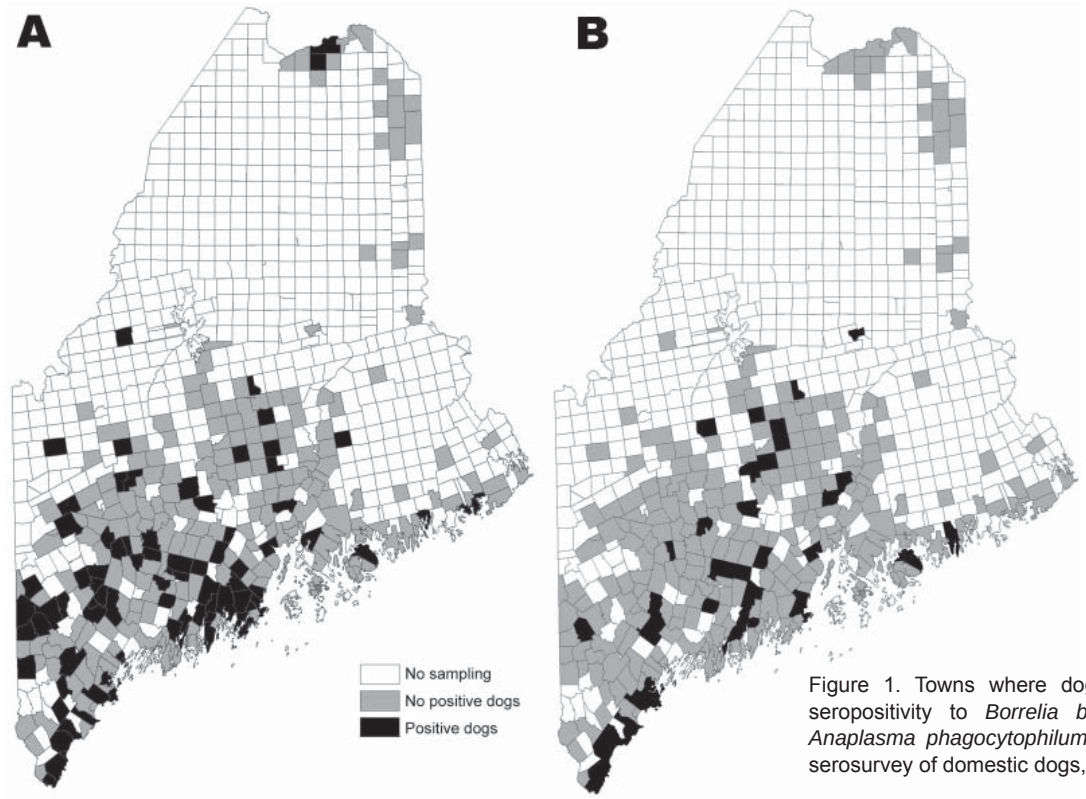

Figure 1. Towns where dogs were tested for seropositivity to *Borrelia burgdorferi* (A) and *Anaplasma phagocytophilum* (B) in a statewide serosurvey of domestic dogs, Maine, USA, 2007.

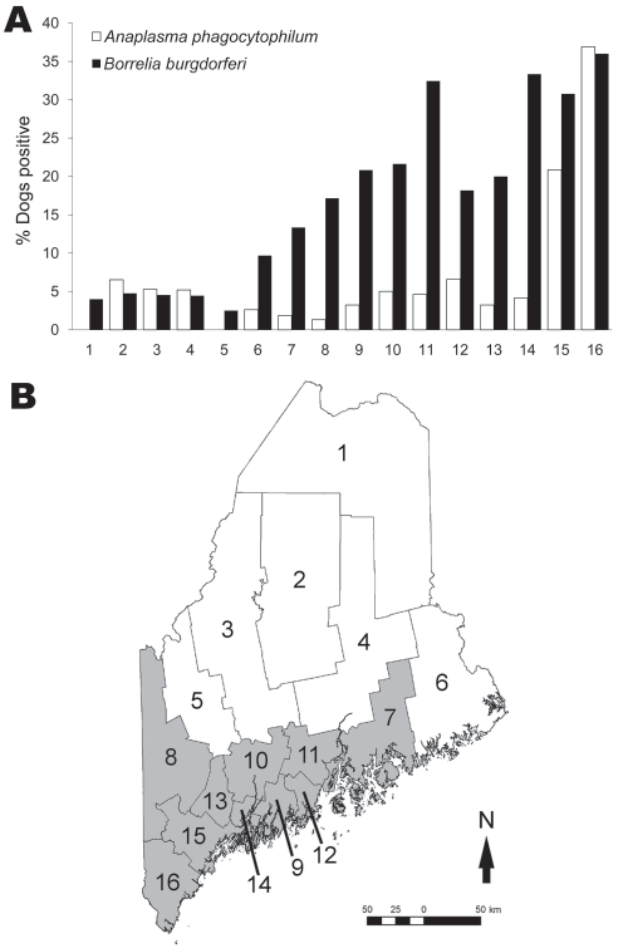

Figure 2. A) Canine seroprevalence for *Anaplasma phagocytophilum* and, in dogs never vaccinated against Lyme disease, for *Borrelia burgdorferi* in Maine counties arranged north to south, 2007. B) Maine counties, with the 10 tick-abundant counties used in the analyses shaded in gray. Counties: 1, Aroostook; 2, Piscataquis; 3, Somerset; 4, Penobscot; 5, Franklin; 6, Washington; 7, Hancock; 8, Oxford; 9, Waldo; 10, Kennebec; 11, Knox; 12, Lincoln; 13, Androscoggin; 14, Sagadahoc; 15, Cumberland; 16, York.

northern counties where it is emerging (63.9% vs. 42.7%; p<0.0001) and correlated positively with the number of deer ticks submitted to our laboratory for identification in 2007 (n = 16 counties, $\rho_{Spearman}$ = 0.63; p = 0.009). Two thirds of respondents said that their dogs had traveled out of town; however, no associations were found between *B. burgdorferi* or *A. phagocytophilum* seropositivity and the dog's travel history. Three of 59 dogs in the northernmost county of Maine were *B. burgdorferi*–positive, 1 of which had never traveled beyond its home town. Eighty-three percent of owners reported using acaricides. Despite the effective protection reported for topical acaricides (*6*,*7*), no difference in seropositivity between treated and untreated dogs was evident on the basis of their reported use (Table 2). Unexplained lameness was 1.5× more likely in dogs that were only *A. phagocytophilum*–positive than in those only *B. burgdorferi*–positive (40.0% vs. 26.5%; p<0.06).

Conclusions

This study demonstrates that risk of contracting Lyme disease has reached northernmost Maine and that anaplasmosis is now being transmitted to dogs throughout the lower half of the state. The study expands on nationwide SNAP 4Dx data documenting *B. burgdorferi* and *A. phagocytophilum* positivity in the southern half of the state (*8*). In southern coastal Maine, overabundant white-tail deer, appropriate habitat, and maritime climate all contribute to high densities of *I. scapularis* ticks (*3*) and consequent disease transmission; thus, the remarkably high level of *A. phagocytophilum* seroreactivity observed in the southern coastal towns of Cape Elizabeth and York calls for further work to understand the dynamics of the intense local emergence of this pathogen. The higher level of unexplained lameness in *A. phagocytophilum*–positive dogs than in *B. burgdorferi*–positive dogs is consistent with findings by Beall et al. (*9*), who reported a 2.6× greater incidence of *A. phagocytophilum* seroreactivity than *B. burgdorferi* seroreactivity among 32 lame, non–co-infected dogs in Minnesota who were suspected of having either disease. The lameness also reflects the high percentage of *B. burgdorferi* positivity among asymptomatic dogs (*10*). That *B. burgdorferi* and *A. phagocytophilum* seropositivity rates were essentially identical between dogs who had a history of travel and those who did not lessens concern about travel as a confounding variable, an exposure difficult to interpret in any event, given the spotty distribution of ticks even where Lyme disease is endemic (*11*).

In a recent study, Hamer et al. (*12*) reported that a serosurvey of canines for *B. burgdorferi* is ineffective in a region that includes areas with little *B. burgdorferi* transmission, and less informative than analysis of ticks removed from dogs. The authors referred to the confounding influence of tick chemoprophylaxis. Our inability to detect an effect of topical acaricides may reflect their ubiquitous use for flea control and a lack of information on the frequency of their use. Although the widespread use of protective measures now complicates interpretation of serosurveys of canines, in selected dogs the availability of a reliable, multitarget test that is used routinely nationwide (*8*) remains a valuable and cost-effective method for documenting transmission of the agents of Lyme borreliosis and anaplasmosis, particularly in areas where disease is emerging.

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We thank Ramaswamy Chandrashekar for assistance with the multitarget test, the staffs of the 47 veterinary clinics for

Table 2. Risk factors vs. canine seroprevalence of *Anaplasma phagocytophilum* and *Borrelia burgdorferi*, Maine, USA, 2007*

Variable	No. dogs	*Borrelia burgdorferi*			*Anaplasma phagocytophilum*		
		No. (%) positive	OR (95% CI)	p value†	No. (%) positive	OR (95% CI)	p value†
Lyme vaccine							
Yes	575	57 (9.9)			49 (8.5)		
No	483	75 (15.3)	1.5 (1.1–2.1)	0.008	23 (4.8)	0.6 (0.3–0.9)	0.02
Travel‡							
All dogs							
None	357	49 (13.7)			27 (7.6)		
≥1	730	89 (12.2)	0.9 (0.6–1.3)	NS	50 (6.9)	0.9 (0.6–1.5)	NS
Unvaccinated dogs							
None	163	21 (12.9)			8 (4.9)		
≥1	320	53 (16.6)	1.3 (0.8–2.3)	NS	15 (4.7)	0.9 (0.4–2.3)	NS
Tick control products							
All dogs							
No	182	20 (11.0)			7 (3.9)		
Yes	899	115 (12.9)	1.2 (0.7–2.0)	NS	66 (7.4)	2.0 (0.9–4.4)	0.08
Unvaccinated dogs							
No	124	13 (10.5)			3 (2.4)		
Yes	350	59 (16.9)	1.7 (0.9–3.3)	0.09	18 (5.1)	2.2 (0.6–7.6)	NS
History of unexplained lameness							
No	877	97 (11.1)			42 (4.8)		
Yes	191	40 (20.9)	2.1 (1.4–3.2)	0.0002	28 (15.2)	3.6 (2.1–5.9)	<0.0001

*A total of 1,087 dogs were tested. OR, odds ratio; CI, confidence interval; NS, not significant.
†Significance based on Pearson χ^2 statistic with 1 degree of freedom.
‡Trips away from town of residence.

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Dr Rand is co-director of the Maine Medical Center Research Institute Vector-borne Disease Laboratory. His primary research interests include the epidemiology and ecology of vector-borne diseases.

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Phylogenetic Analysis of West Nile Virus Isolates, Italy, 2008–2009

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To determine the lineage of West Nile virus that caused outbreaks in Italy in 2008 and 2009, several West Nile virus strains were isolated from human specimens and sequenced. On the basis of phylogenetic analyses, the strains isolated constitute a distinct group within the western Mediterranean cluster.

West Nile virus (WNV) is an arthropod-borne flavivirus affecting a wide range of vertebrates, including birds and mammals. The natural cycle of infection involves birds and mosquitoes (*1*); many species of wild birds act as amplifying hosts, whereas humans and horses are considered dead-end hosts (*2*). First identified in tropical Africa, WNV infection has been found in northern Africa, Israel, India, and Australia (*3*) and has progressively spread in the Americas since 1999 (*4*,*5*). WNV has been the cause of outbreaks and sporadic cases in central, eastern, and Mediterranean Europe for >45 years (*6*). Most strains responsible for the European and Mediterranean basin outbreaks are in lineage 1, with most grouped in the so-called European Mediterranean/Kenyan cluster (*7*,*8*).

In August 2008, an outbreak involving wild birds, horses, and humans affected 8 provinces in 3 regions in Italy (Emilia-Romagna, Veneto, Lombardy) (*9*,*10*). In 2009, a new epidemic was reported in the same region and in other neighboring regions in Italy, with up to 17 confirmed human cases of West Nile neuroinvasive disease (*11*).

The Study

We obtained nucleotide sequences of the envelope (E) and nonstructural (NS)3/NS5 protein regions of 6 WNV human strains isolated during the 2008–2009 outbreaks in Italy and analyzed their phylogenetic relationships with other WNV strains isolated in Europe and the Mediterranean basin. Virus isolation on Vero E6 cells was performed from human serum samples obtained from patients with neuroinvasive WNV infection: 5 samples were collected during the 2009 outbreak from patients residing in the provinces of Ferrara (2 patients), Modena (1), and Mantua (2); 1 sample was collected during the 2008 outbreak from a patient living in Ferrara. Cytopathic effects were observed 6 days after the serum was injected into cell cultures; WNV infection was identified by immunofluorescence staining and confirmed by real-time reverse transcription–PCR (*9*).

Virus isolates were designed as follows: TOS-09 (Ferrara), NAL-09 (Ferrara), CHI-09 (Mantua), BAL-09 (Mantua), FAN-09 (Modena), and MAN-08 (Ferrara). After 1 passage, supernatants of infected tissue cultures were processed for viral RNA extraction and PCR amplification. Nucleotide sequences were obtained for the E gene (1,503 nt) and for a region of 5,759 nt encompassing the NS3, NS4a, NS4b, and NS5 genes, which are considered to be the most phylogenetic informative regions (*12*,*13*). Overlapping amplicons were amplified and bidirectionally sequenced. Primer sequences are available upon request.

Multiple alignments of the 6 new WNV sequences of E and NS3/NS5 regions, along with the corresponding sequences from 30 other WNV strains and isolates available in GenBank and Usutu virus as outgroup virus, were generated by using ClustalW software (www2.ebi.ac.uk/clustalw). Phylogenetic analyses of the E (Figure 1) and NS3/NS5 (Figure 2) sequences generated highly comparable topologies and confirmed that strains from Italy and all western Mediterranean strains belonging to lineage 1, clade 1a, were highly related. In addition, on the basis of the phylogenetic trees of E and NS3/NS5 sequences, we found that the 6 new sequences clustered with other strains isolated in Italy in 2008 and 2009, constituting a distinct and well-supported group within the western Mediterranean cluster (100% bootstrap on E and NS3/NS5 regions) (Figures 1, 2).

For the E gene, the mean genetic distance within the 6 new isolates from Italy was 0.08% at the nucleotide level, whereas the derived amino acid sequences were totally conserved. For the NS3/NS5 region, the mean genetic distance within the 6 new isolates from Italy was 0.10% at the nucleotide and amino acid levels.

Within the 2008–2009 cluster from Italy, the 6 newly sequenced human strains exhibited a 0.14% mean nucleotide distance with the 2 strains isolated in the Emilia Romagna region in 2008 from magpies (Italy 15803/08 and Italy 15217/08) and 0.15% with the first human WNV strain isolated in Italy in 2009 (Italy/09) in the Veneto region (*14*),

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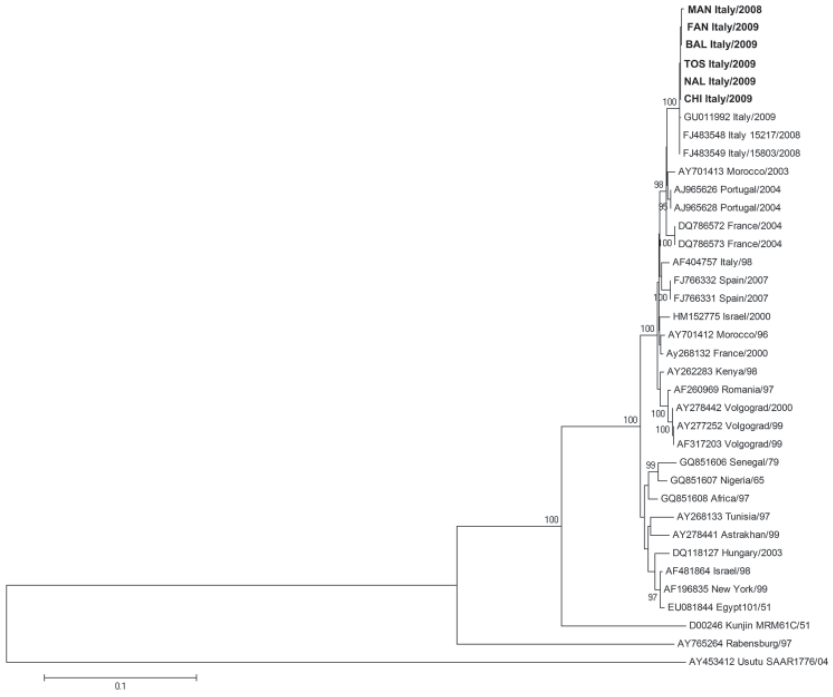

Figure 1. Phylogenetic trees of West Nile virus strains isolated during outbreaks in Italy, 2008–2009, based on nucleotide sequences of the complete envelope gene. Phylogenetic tree and distance matrices were constructed by using nucleotide alignment, the Kimura 2-parameter algorithm, and the neighbor-joining method implemented in MEGA version 4.1 (www.megasoftware.net/mega4/mega41.html). The tree was rooted by using Usutu virus as the outgroup virus. The robustness of branching patterns was tested by 1,000 bootstrap pseudoreplications. The percentage of successful bootstrap replicates is indicated at nodes, showing only values >80. The scale bar indicates nucleotide substitutions per site. Strains sequenced in this study are TOS-09, FAN-09, BAL-09, CHI-09, NAL-09, and MAN-08. Envelope nucleotide sequence GenBank accession nos: NAL-09, HM991272; TOS-09, HM991273; BAL-09, HM991274; CHI-09, HM991275; MAN-09, HM991276; FAN-09, HM991277. **Boldface** indicates the 6 newly sequenced strains.

in the E and NS3/NS5 genomic regions. On the basis of NS3/NS5 sequences, all strains from Italy isolated during the 2008–2009 outbreaks showed the highest relatedness to Morocco/03, France/04, and Portugal/04 with 1.08%, 1.28%, and 1.45% mean nucleotide distance, respectively, whereas they show a mean nucleotide distance of 1.56% with Italy/98. The close relatedness to Morocco/03, France/04, and Portugal/04 was also confirmed on E gene sequences (1.44%, 1.51%, and 1.17% mean nucleotide distance, respectively), compared with a mean nucleotide distance of 2.06% with Italy/98.

Comparison of the amino acid sequences of the E and NS3/NS5 region showed that all 2008–2009 strains from Italy exhibit a set of amino acid substitutions in the NS4a and NS5 proteins not shared with other western Mediterranean strains that could be considered as molecular signatures, e.g., I2209 and S2224 in the NS4a protein, A2786 and K2950 in the NS5 protein (online Appendix Table, www.cdc.gov/EID/content/17/5/903-appT.htm). No relevant amino acid changes were observed in the E region.

Notably, the NS3 Thr249Pro substitution, suggested to be associated with increased virulence for American crows (*15*), was not uniformly present in all the 2008–2009 isolates in Italy. It was detected in all 2008 isolates, but heterogeneously distributed among the 2009 isolates. In particular, among the newly sequenced strains, P at NS3–249 was present in the BAL-09, FAN-09, and MAN-08 isolates, whereas T was present in the TOS-09, NAL-09, and CHI-09 isolates. Thus, this substitution could not be considered a hallmark of the well-defined 2008–2009 cluster in Italy.

Conclusions

Although in the past decades several outbreaks of WNV infection have occurred in Europe and in the Mediterranean Basin (*6*), few viral isolates and nucleotide sequences are available from this geographic area, thus preventing an in-depth analysis of the molecular epidemiology of West Nile infection in the region. We provide 6 new nucleotide sequences of the E and NS3/NS5 regions from WNV strains isolated in 2008–2009 from patients in Italy with West Nile neuroinvasive disease and describe the isolates' phylogenetic relationships with other WNV strains in the Western Mediterranean cluster.

The analysis of the E and NS3/NS5 sequences enabled a new phylogenetic reconstruction of the Western Mediterranean cluster, suggesting that the 2008–2009 strains from Italy constitute a distinct and well-supported group within the European–Mediterranean clade, characterized by specific amino acid changes in NS4a and NS5 proteins. The structure of phylogenetic trees, together with the presence of amino acid signatures, is consistent with a common origin of the strains circulating 2008–2009, supporting the concept of endemic circulation of WNV in Italy (*6*).

Furthermore, the T249P change in WNV-NS3 helicase has been suggested as increasing the virulence of WNV in birds in North America (*15*), although recent data do not

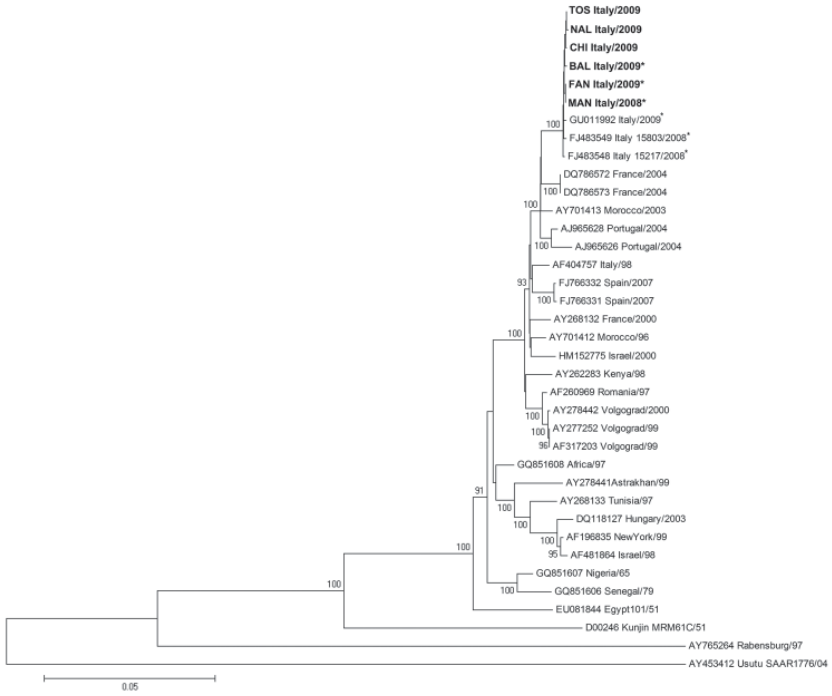

Figure 2. Genomic region encompassing nonstructural (NS) 3, NS4a, NS4b, and NS5 genes. Phylogenetic tree and distance matrices were constructed by using nucleotide alignment, the Kimura 2-parameter algorithm, and the neighbor-joining method implemented in MEGA version 4.1 (www.megasoftware.net/mega4/mega41.html). The tree was rooted by using Usutu virus as the outgroup virus. The robustness of branching patterns was tested by 1,000 bootstrap pseudoreplications. The percentage of successful bootstrap replicates is indicated at nodes, showing only values >80. The scale bar indicates nucleotide substitutions per site. Strains sequenced in this study are TOS-09, FAN-09, BAL-09, CHI-09, NAL-09 and 191 MAN-08. NS3/NS5 region nucleotide sequence GenBank accession nos.: NAL-09, HM641230; TOS-09, HM641225; BAL-09, HM641226; CHI-09, HM641227; MAN-09, HM641229; FAN-09, HM641228. **Boldface** indicates the 6 newly sequenced strains. *Strains from Italy carrying the T249P mutation.

support this hypothesis (*8*). Our results indicate that, at least in 2009 in Italy, there was a co-circulation of WNV strains carrying either a P or a T in this position. On the basis of our study, it is not possible to establish whether this was because of different virus introductions in Italy or reversion of some originally mutated strains. However, our findings do not support the hypothesis that the recent emergence of this mutation might have contributed to rapid spread of WNV infection with the occurrence of human cases in the Mediterranean Basin. More accurate studies are required to define the role of the T249P mutation in the pathogenesis and evolutionary history of WNV in recent outbreaks.

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Genomic Characterization of Nipah Virus, West Bengal, India

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An intrafamilial outbreak in West Bengal, India, involving 5 deaths and person-to-person transmission was attributed to Nipah virus. Full-genome sequence of Nipah virus (18,252 nt) amplified from lung tissue showed 99.2% nt and 99.8% aa identity with the Bangladesh-2004 isolate, suggesting a common source of the virus.

Nipah virus (NiV) causes encephalitis or respiratory signs and symptoms in humans, with high death rates (*1–4*). NiV outbreaks have been reported from Malaysia, Bangladesh, Singapore, and India (*1*,*5–13*). Cases in humans have been attributed to zoonotic transmission from pigs and bats (*1*,*14*). We describe a full genome sequence of NiV from an outbreak in India.

The Study

During April 9–28, 2007, five persons became ill and died within a few days at village Belechuapara, Nadia district, West Bengal, India, which borders Bangladesh. The index case-patient (case-patient 1) was a 35-year-old male farmer addicted to country liquor derived from palm juice. Hundreds of bats were observed hanging from the trees around his residence, which strongly suggested association with the infection of the index case-patient and possibility of contamination of the liquor with bat excreta or secretions.

Three patients were close relatives of case-patient 1: his 25-year-old brother (case-patient 2), his 30-year-old wife (case-patient 3), and his 39-year-old brother-in-law (case-patient 4). They became ill 12, 14, and 14 days, respectively, after contact with case-patient 1. In another person, a 28-year-old man (case-patient 5) who collected blood samples from and performed a computed tomography scan of the brain of case-patient 1, symptoms developed 12 days after contact.

No samples were available from the first 2 case-patients. Brain and lung tissues from case-patient 3, cerebrospinal fluid (CSF) from case-patient 4, and urine and CSF from case-patient 5 were collected. Blood samples were obtained from case-patients 4 and 5 and from 34 asymptomatic contacts from the village. Serum samples from these persons were tested for immunoglobulin (Ig) M and IgG antibodies to NiV (IgM/IgG anti-NiV) with ELISA by using reagents provided by the Centers for Disease Control and Prevention (Atlanta, GA, USA). To detect NiV RNA, urine (250 μL), CSF (100 μL), or autopsied brain or lung tissue (100 mg) were used, and RNA was extracted by using TRIzol LS and TRIzol reagents (Invitrogen Life Technologies, Carlsbad, CA, USA). Nested reverse transcription–PCR (RT-PCR) was conducted by using nucleocapsid (N) gene–based primers (*8*).

Attempts to isolate NiV in Vero E6 cell lines or infant mice were unsuccessful. The full-length genomic sequence was obtained from the lung of case-patient 3 by using 36 sets of primers (online Appendix Table, www.cdc.gov/EID/content/17/5/907-appT.htm), Superscript II RNase reverse transcriptase for reverse transcription (Invitrogen), and Pfx polymerase for amplification (Invitrogen). The PCR products of predicted molecular size were gel eluted (QIAquick PCR Purification Kit; QIAGEN, Hilden, Germany) and sequenced by using BigDye Terminator cycle sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and an automatic Sequencer (ABI Prism 3100 Genetic Analyzer; Applied Biosystems). PCR products were sequenced in both directions. To determine the genotypic status, phylogenetic analysis was conducted by using partial N gene and full NiV genome sequences with the Kimura 2-parameter distance model and neighbor-joining method available in MEGA version 3.1 software (www.megasoftware.net). The reliability of phylogenetic groupings was evaluated by the bootstrap test with 1,000 bootstrap replications.

Patients' signs and symptoms included high fever (103°F–105°F [39.4°C–49.6°C]) with and without chills, severe occipital headache, nausea, vomiting, respiratory distress, pain in calf muscles, slurred speech, twitching of facial muscles, altered sensorium, (focal) convulsions, unconsciousness, coma, and death. The first 3 case-patients died within 2–3 days after symptom onset; case-patients 4 and 5 died after 5 and 6 days, respectively. Clinical investigations could be conducted for case-patients 4 and 5. Results of serologic tests for malaria parasite, typhoid, anti-dengue IgM, HIV, and hepatitis B surface antigen were negative. Peripheral blood profiles were within

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reference limits. Alanine aminotransferase, aspartate aminotransferase, creatine phosphokinase, and C-reactive protein were elevated. Blood gas analysis in case-patient 4 (case-patient 5 values in parentheses) showed oxygen saturation 49.4% (71%), pO_2 36.1 mm Hg (44.8 mm Hg), pCO_2 44.4mm Hg (44.1 mm Hg), HCO_3 15.7 mmol/L (19.1 mmol/L), and pH 7.166 (7.255). Lumbar puncture of case-patient 5 showed opening pressure within reference range (1 drop/second), 2 cells/cm; all cells observed were lymphocytes. Chest radiograph indicated pulmonary edema, which suggested acute respiratory distress syndrome. At the time of admission, computed tomography and magnetic resonance imaging scans of the brain showed no abnormality.

Serum samples from case-patients 4 and 5 were positive for IgM anti-NiV. Of the clinical samples screened, brain and lung tissues of case-patient 3, CSF of case-patient 4, and urine of case-patient 5 were NiV RNA positive. Of the 34 asymptomatic contacts, 1 was positive for IgG anti-NiV and negative for IgM anti-NiV and did not report any major illness in the past. This positivity may reflect a previous subclinical infection or cross-reactivity in ELISA needing further follow-up.

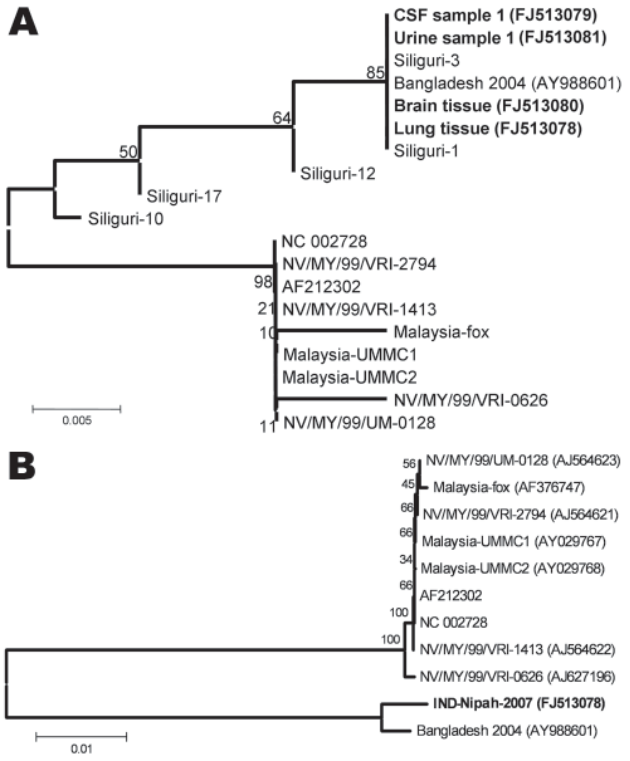

Figure. A) Phylogenetic analysis based on partial nucleocapsid (N) gene nucleotide sequences (159 nt, according to Nipah virus [NiV] Bangladesh sequence, GenBank accession no. AY988601, 168–327 nt) of the 4 NiVs sequenced during this study (**boldface**). Five sequences of the viruses from Siliguri (*8*) and from representative NiV sequences obtained from GenBank indicated by the respective accession numbers. Values at different nodes denote bootstrap support. B) Full genome–based phylogenetic analysis of the NiV sequenced from the lung tissue of a patient (**boldface**). Representative NiV sequences obtained from GenBank are indicated by the respective accession numbers. Values at different nodes denote bootstrap support. Scale bars indicate nucleotide substitutions per site.

Before this report, similar cases had not been reported from the village or the surrounding area. Partial N gene sequences confirmed NiV in the clinical specimens from all 3 case-patients. Phylogenetic analysis showed that similar to findings from the 2001 outbreak study (*8*), viruses from Bangladesh and India clustered and diverged from the viruses from Malaysia. (Figure, panel A). The length of the full genome of the isolate from India was 18,252 nt. The sequence of this virus (INDNipah-07–1, GenBank accession no. FJ513078) was closer to the virus from Bangladesh (Figure, panel B), with 99.2% (151 nt substitutions) and 99.80% (17 aa substitutions) identity at nucleotide and amino acid levels respectively. Of the 151 nt substitutions, 9 occurred in the N open reading frame (ORF),11 in the phosphoprotein ORF, 8 in the matrix ORF, 11 in the fusion glycoprotein ORF, 7 in the attachment protein ORF, and 47 in the large polymerase ORF. Fifty-eight substitutions occurred in nontranslated regions at the beginning and the end of each ORF. The intergenic sequences between gene boundaries were highly conserved in the isolate from India, compared with the isolate from Bangladesh, which showed 1 change (GAA to UAA) between the attachment protein and large polymerase genes. No change was observed in the leader and the trailer sequences.

The Table compares amino acid substitutions in the different regions of the genome of the isolate from India with those of the viruses from Bangladesh and Malaysia. Of the 17 aa substitutions, 7 were unique to the isolate from India, and 10 were similar to the isolates from Malaysia. Overall, however, the isolate from India was closer to the isolate from Bangladesh, although distinct differences were observed.

To our knowledge, this is the second report of an NiV outbreak in India, identified within 1 week of the investigation. The first outbreak affected mainly hospital staff or persons visiting hospitalized patients; the 74% case-fatality rate strongly suggested person-to-person transmission (*8*). Both outbreaks (2001 and 2007) occurred in the state of West Bengal bordering Bangladesh wherein several outbreaks of the disease have been reported (*7*,*9*–*13*). However, fruit bats from West Bengal have not been screened for evidence of NiV infection. This state needs to create awareness about NiV and obligatory testing of suspected case-patients.

Conclusions

NiV caused an intrafamilial outbreak with a 100% case-fatality rate, which confirmed person-to-person

Table. Regionwise amino acid substitutions in the Nipah virus genome*

Region and amino acid position	India	Bangladesh	Malaysia
Phosphoprotein			
228	**K**	R	R
276	**S**	G	G
285	R	H	R
310	**R**	G	G
Nucleocapsid protein			
188	E	D	E
211	**R**	Q	Q
Matrix protein			
13	**I**	M	M
Fusion protein			
19	**I**	M	M
207	L	S	L
252	D	G	D
Attachment protein			
304	**V**	I	I
Large polymerase protein			
94	I	T	I
112	K	R	K
632	N	S	N
639	N	D	N
665	T	I	T
1748	I	V	I

*GenBank accession numbers of isolates examined: India (FJ513078), Bangladesh (AY988601), and Malaysia (AY029767,AY029768, and AJ564623). **Boldface** indicates unique amino acids in the isolate from India.

transmission. The NiV strains from India and Bangladesh were closer than the Malaysian viruses. Although the outbreaks occurred in neighboring geographic areas, NiV outbreaks in Bangladesh and India were not caused by the same virus strain or by spillover.

Acknowledgments

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Chikungunya Virus, Southeastern France

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In September 2010, autochthonous transmission of chikungunya virus was recorded in southeastern France, where the *Aedes albopictus* mosquito vector is present. Sequence analysis of the viral genomes of imported and autochthonous isolates indicated new features for the potential emergence and spread of the virus in Europe.

Chikungunya virus (CHIKV; *Togaviridae*, genus *Alphavirus*), transmitted to humans by the bite of *Aedes* spp. mosquito, leads to an acute fever associated with an arthromyalgic syndrome (*1*). CHIKV outbreaks occurred after the virus's recent expansion in Africa, the Indian Ocean, India, and Southeast Asia. Phylogenetic analyses have demonstrated 3 distinct lineages of CHIKV strains: West Africa, Asia, and East/Central/South Africa (ECSA) (*2*,*3*). Strains from the Indian Ocean and India segregate into 2 independent sublineages that presumably derive from an East African ancestral genotype (*2*,*3*). Until recently, the *Ae. aegypti* mosquito was widely accepted as the main urban vector of CHIKV. However, the *Ae. albopictus* mosquito was extensively implicated in CHIKV transmission during the 2005–06 outbreak in Réunion Island (*1*).

The Study

Reinforced surveillance systems aimed at monitoring the introduction of CHIKV have been implemented in 6 departments in southeastern France, including the Var department, where *Ae. albopictus* has spread since its introduction in 2004, presumably from northern Italy (*4*). On August 29, 2010, a 7-year-old girl (patient 1) with acute febrile syndrome, headache, and abdominal pain sought treatment in the city of Fréjus (Var) 1 day after she had returned from Rajasthan, India. Continuous CHIKV circulation in northern India districts has been reported during 2009–2010 (www.promedmail.org). The patient's serum sample was found positive for CHIKV infection by reverse transcription–PCR (RT-PCR) (*5*,*6*). Three weeks after the notification of patient 1, another young girl (patient 2) experienced clinical symptoms that began on September 18 with fever, arthralgia, backache, headache, and retro-orbital pain. Patient 2 had no history of travel in areas endemic for CHIKV. She resided 2.5 km from patient 1. The serum specimen was positive for CHIKV diagnosis. Patient 2's physician reported that a young girl (patient 3), a close friend of her patient, showed clinical symptoms compatible with CHIKV infection at the same time. Patient 3, who lives near patient 1, had invited patient 2 to spend the night of September 15 at her home. The 2 children reported numerous mosquito bites. A serum sample from patient 3 was collected 1 week after onset of fever and monoclonal antibody capture ELISA detected high titers of specific anti-CHIKV immunoglobulin M. The serum sample also showed a weak RT-PCR signal for CHIKV. Given that patients 2 and 3 did not report any recent travel to areas endemic for CHIKV, their illnesses were classified as autochthonous cases of CHIKV infection. No complications were recorded, but all 3 patients had persistent weakness and joint pain 3 months after the acute phase.

High densities of *Ae. albopictus* mosquitoes have been found in the Var department since 2008. Intensive mosquito control measures, including spraying for adult mosquitoes and destroying breeding sites, were undertaken around the patients' residences and areas visited by confirmed case-patients. No further cases were found by the active case finding system (a local physician and laboratories network) implemented for 45 days after the declaration of the last autochthonous case.

A molecular study of France/2010 CHIKV strains isolated in Fréjus obtained from patients 1 (imported case) and 2 (autochthonous case) was performed. Viral genomic RNA was extracted from CHIKV grown once in mosquito C6/36 cells and then subjected to RT-PCR amplification by using a set of primers targeting the structural genes of CHIKV (*7*). Paired sequence analysis of the E2–6K–E1 junction showed that the 2 France/2010 CHIKV strains display a divergence rate <0.05% at the nucleotide level, whereas 100% identity was observed at the amino acid level. Phylogenetic analysis demonstrated that these viral strains belong to a cluster that is closely related to strains from India within the ECSA lineage (Figure). The France/2010 CHIKV isolate from patient 2 might be derived from an Indian strain introduced by patient 1 (index case). Genotypes E2-211T, E2-312M, E2-386A, 6K-8I, and E1-284E that are found in the currently circulating

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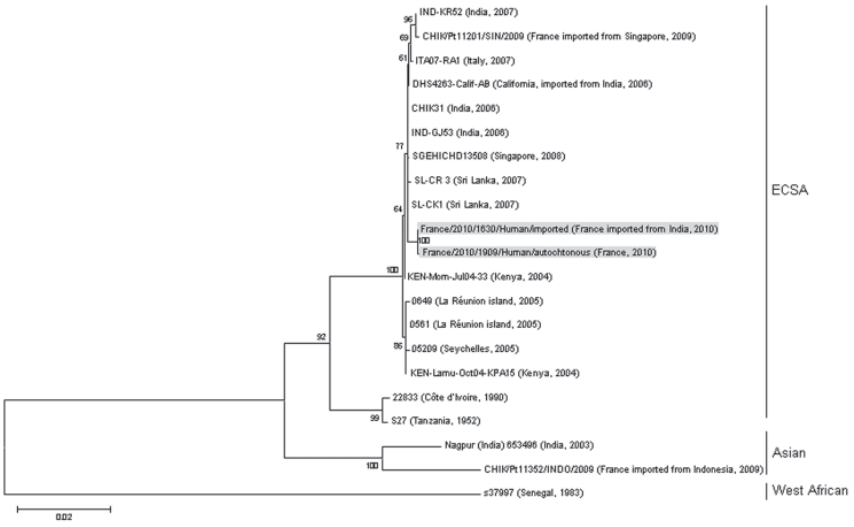

Figure. Phylogenetic relationships among chikungunya virus isolates from cases of chikungunya fever in France, based on complete E2-6K-E1 nucleotide sequence (2,771 nt) analysis. Gray shading indicates imported and autochthonous strains. Sequence alignments were performed by using BioNumerics version 5.1 (www.applied-maths.com). Phylogenetic analysis was inferred by using the maximum-likelihood method as implemented in MEGA version 5 software (www.megasoftware.net). Bootstrap support values (1,000 replicates) are indicated at major nodes. The sequence of the strains from France described in this study has been deposited in GenBank (accession number pending); other sequences were retrieved from GenBank. Scale bar indicates number of base substitutions per site. ECSA, east/central/south Asia.

strains belonging to the ECSA lineage were identified in France/2010 CHIKV isolates (*2*,*3*,*7*). These isolates also display the genotype E1-211E specifically shared by viral strains belonging to the Asian phylogenetic group (Table). The residue Ala at position E2-264 has not been previously described in any CHIKV strains.

Recent attention has focused on the predominant role of E1 and E2 proteins in successful CHIKV infection of the

Table. Relevant amino acid substitutions identified between France/2010 CHIKV isolates (autochthonous and imported cases) versus a selection of CHIKV strains*

			Protein position							
Strain	Country	Year	E2-60	E2-162	E2-211	E2-264†	E1-211	E1-226	E1-269	E1-284
France/2010/1630/human/imported (a)	France (India)	2010	D	A	T	**A**	**E**	**A**	V	E
France/2010/1909/human/autochthonous (a)	France	2010	D	A	T	**A**	**E**	**A**	V	E
22833 (a)	Côte d'Ivoire	1990	D	A	I	V	K	A	M	D
CHIK31 (a)	India	2006	D	A	T	V	K	A	V	E
05209 (a)	Seychelles	2005	D	A	T	V	K	A	V	E
0561 (a)	Réunion	2005	D	A	T	V	K	A	V	E
0649 (a)	Réunion	2005	D	A	T	V	K	V	V	E
DHS4263-Calif-AB (a)	California (India)	2006	D	A	T	V	K	A	V	E
IND-GJ53 (a)	India	2006	D	A	T	V	K	A	V	E
IND-KR52 (a)	India	2007	D	A	T	V	K	V	V	E
ITA07-RA1 (a)	Italy	2007	D	A	T	V	K	V	V	E
KEN-Lamu-Oct04-KPA15 (a)	Kenya	2004	D	A	T	V	K	A	V	E
KEN-Mom-Jul04-33 (a)	Kenya	2004	D	A	T	V	K	A	V	E
S27 (a)	Tanzania	1952	D	A	I	V	K	A	M	D
SGEHICHD13508 (a)	Singapore	2008	D	A	T	V	K	A	V	E
CHIK/Pt11201/SIN/2009 (a)	France (Singapore)	2009	D	A	T	V	K	V	V	E
SL-CK1 (a)	Sri Lanka	2007	D	A	T	V	K	A	V	E
SL-CR 3 (a)	Sri Lanka	2007	D	A	T	V	K	A	V	E
Nagpur (India) 653496 (b)	India	2003	D	A	T	V	E	A	M	D
CHIK/Pt11352/INDO/2009 (b)	France (Indonesia)	2009	D	A	T	V	E	A	M	D
s37997 (c)	Senegal	1983	D	A	T	V	K	A	V	D

*Molecular signatures were based on the analysis of complete amino acid sequence E2-6K-E1 (923 aa). The numbering of amino acid positions refers to the African isolate S27 (GenBank access no. AF369024). Residues in **boldface** indicate critical aa changes. Letters in parentheses after strain names refer to East/Central/South Africa (a), Asia (b) and West Africa (c) phylogroups. Country names in parentheses identify source of imported case. CHIKV, chikungunya virus.
†The amino acid substitution was unique to France/2010 CHIKV isolates.

anthropophilic *Ae. albopictus* (*2*,*3*,*7*,*8*). Vector competence experiments with La Réunion/2006 CHIKV isolates demonstrated the importance of the newly acquired E1-Ala226Val substitution for efficient transmission by *Ae. albopictus* mosquitoes during the 2006 outbreak in Réunion Island (*7*–*10*). Italy/2007 CHIKV strains also exhibited the signature E1-226V genotype (*11*). *Ae. albopictus* from northern Italy and from southeastern France showed disseminated infection rates ranging from 75%–90% for CHIKV strains with E1-226V (*10*). The 2 France/2010 CHIKV strains isolated in Fréjus have Ala at position E1-226 (Table). The presence of an Asp residue at position E2-60, found in most of the ECSA CHIKV strains, may in part counterbalance the less favorable transmission of E1-226A strain in *Ae. albopictus* (Table). The Thr residue at position E2-211 potentiates the infectivity of CHIKV in *Ae. albopictus* mosquitoes only in synergy with E1-226V. The presence of E2-211T in CHIKV isolates from France underlines the risk for emergence of a fully adapted viral variant if the E1-226V genotype was selected during continuous transmission within *Ae. albopictus* populations in France (*7*,*8*,*9*).

Conclusions

The efficient CHIKV transmission in Italy and southeastern France sheds new light on its dissemination potential in Europe from 1 index case, regardless of the viral genetic background and mosquito species in the region of origin of the imported CHIKV (*1*,*10*,*11*). In emerging regions, such as Italy and Réunion Island, where the seroprevalence in the population was <50%, no confirmed cases were recorded for years after an outbreak. Italy has not reported any new autochthonous cases since 2007. However, >2 years passed since the end of the epidemic in Réunion Island before a local transmission of CHIKV was again detected. In Europe where sylvatic cycles are absent, vertical transmission may participate in the maintenance and/or cyclic reemergences of CHIKV. This critical issue remains to be investigated in diapausing temperate populations of *Ae. albopictus* that may have more efficient vertical transmission than mosquito populations in eastern Italy and tropical regions (*11*,*12*).

In 2010, southeastern France faced the concomitant emergences of dengue virus (DENV) and CHIKV (*13*). For each of these viruses, only 2 autochthonous infections were confirmed, which suggests that rapid detection and control measures implemented around imported and autochthonous cases have been efficient. A recent report mentioned the dual emergence of CHIKV and DENV in southeastern France and urged the implementation of specific surveillance and response measures to reduce the risk for arbovirus emergence (*14*). Since 2006, a specific chikungunya/dengue national preparation and response plan based on rapid detection and investigation of imported and suspected autochthonous cases, mosquito control measures, and efficiency evaluation in the treated areas has been activated from May through November and then modified after annual debriefing meetings involving all partners. In 2010, this model proved to be well adapted to the early detection and control of CHIKV and DENV. Considering the expanding global distribution of *Ae. albopictus* mosquitoes and the successful emergence of CHIKV in Italy and France, reinforced surveillance and response to CHIKV and DENV dissemination should become a higher priority in Europe (*15*).

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Upward Trend in Dengue Incidence among Hospitalized Patients, United States

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International travel and a global expansion of dengue fever have the potential to increase the incidence of dengue in the United States. We conducted a retrospective cohort analysis of trends in dengue among hospitalized patients by using the National Inpatient Sample (2000–2007); the number of cases more than tripled (p<0.0001).

The worldwide number of cases of dengue infection has increased nearly 35-fold in the past half-century, with a concomitant rapid geographic expansion (*1*). A large percentage of the world's population is at risk for dengue fever: an estimated 2.5 billion persons live in virus-endemic areas. Each year, 50–100 million cases occur, hospitalizations for the infection have reached 500,000, and the global death toll is >20,000 persons (*2*). Large outbreaks have also occurred in close proximity to the US mainland (*3*,*4*). Despite the close proximity of these outbreaks to the United States, autochthonous cases in the continental United States have been relatively unusual, until the recent large autochthonous outbreak in Florida (*5*,*6*).

Risk for dengue infection to US residents has primarily been posed by travel. Among a multinational sample of ill travelers with a systemic febrile illness for whom a diagnosis could be determined, the GeoSentinel Surveillance Network reported that dengue fever was the second most common cause of such cases, behind malaria (*7*). A study from the same network reported that among travelers from all but 2 regions confirmed or probable dengue was more common than malaria (*8*). However, a recent published report indicated that, although reported cases of travel-associated dengue had increased during 1996–2005, "no significant trend" was shown (*9*). Another recent report showed a travel-associated increase, but this finding may have been due to, in part, the expansion of surveillance to include 2 independent monitoring systems (*10*). Because dengue has not been a reportable disease in the United States until recently, incidence and disease trends are difficult to determine. The goal of this study was to determine incidence of dengue fever among hospitalized patients and to analyze the recent trend in hospitalizations among patients with this disease.

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The Study

We conducted a retrospective cohort analysis of trends in dengue diagnoses among hospitalized patients using the National Inpatient Sample, the largest all-payer database of hospital discharges in the United States. The database is maintained as part of the Healthcare Cost and Utilization Project by the Agency for Healthcare Research and Quality and consists of a 20% stratified sample of discharges from nonfederal acute care hospitals (*11*). We first extracted all discharges from the National Inpatient Sample for hospital admission (i.e., the denominator of the incidence rate) from 2000 through 2007. Among this population, we then identified cases of dengue fever coded as either a primary or secondary diagnosis (i.e., code 061, dengue fever), according to the International Classification of Diseases, Ninth Revision, Clinical Modification.

For each yearly incidence rate, we calculated a 95% exact binomial confidence interval. To determine whether a significant trend in hospitalizations of patients with dengue fever occurred during the study period, we fit a logistic regression model using yearly incidence as the dependent variable and year as the independent variable. In addition, to accommodate the temporal association in the yearly incidence, we fit the model using generalized estimating equations, assuming an autoregressive correlation structure.

We also calculated the ratio of the yearly incidence rates at the beginning and the end of the study period (i.e., rates in 2000 and 2007). We tested whether this incidence ratio is significantly different from one using the Fisher exact test and computed a 95% exact confidence interval for the corresponding odds ratio using the hypergeometric distribution. (Because the incidence rates are fairly low, the odds ratio closely approximates the incidence ratio.)

Finally, we used the Monte Carlo variant of the Fisher exact test to investigate possible geographic variation in the incidence rate among the 4 US census regions. We tested for geographic homogeneity for every year in our 8-year sample. All statistical analyses were performed by using R version 2.10.1 (R Foundation for Statistical Computing; www.r-project.org) and SAS version 9.2 (SAS Institute Inc., Cary, SC, USA.).

During 2000–2007, ≈1,250 patients were hospitalized for dengue fever. The mean age of this population was 38 years (range newborn–87 years). The length of stay for these patients ranged from 0 to 35 days (median 3 days). For the Monte Carlo variant of the Fisher exact test, we

found the incidence rates for the 4 US Census regions were homogenous for all years, except for 2004 and 2007. In these 2 years, the Northeast Region had the highest incidence rate ($p<0.0001$ for each year). Over the study period, the estimated number of dengue cases more than tripled from 81 cases in 2000 to 299 cases in 2007. The trend in the incidence of patients hospitalized with dengue during the study period was upward and significant (trend estimate 0.1313, model-based SE 0.0258; $p<0.0001$) (Figure). The increase from 2000 to 2007 was also significant (incidence ratio 3.5641, 95% confidence interval 2.0293–6.6232; $p<0.0001$).

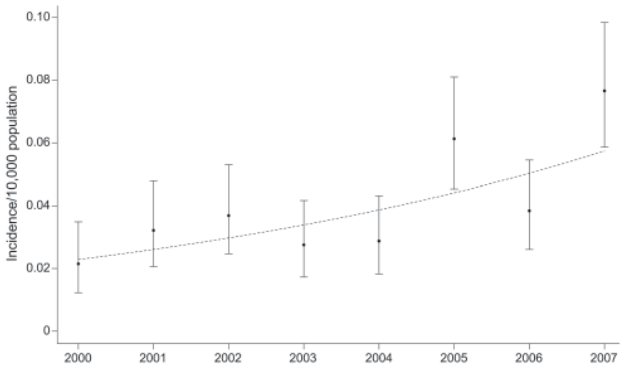

Figure. National estimates of dengue yearly incidence rates and 95% exact binomial confidence intervals (error bars), calculated by using data from the National Inpatient Sample, United States, 2000–2007. The trend (dotted line) is based on a logistic regression model fit by using generalized estimating equations. Note that the trend is curvilinear in the incidence rate, yet linear in the log odds of the incidence.

Conclusions

We found a dramatic increase in the number of hospitalizations for patients with dengue fever in the United States. This increase is not surprising considering that 1) the number of cases in disease-endemic regions has increased in recent years, and 2) a substantial number of travelers annually enter the United States from the tropics and subtropics (*12*).

Although infrequent, severe consequences of dengue infection may occur in returning travelers. As individual travelers increasingly make multiple visits to dengue-endemic areas, the risk for severe dengue infections may similarly increase. A survey of 219 travelers who received treatment for dengue in Europe showed that 23 (11%) had severe clinical manifestations, including internal hemorrhage, plasma leakage, shock, and marked thrombocytopenia (*13*). We were unable to ascertain whether mosquito-borne hemorrhagic fever (International Classification of Diseases, Ninth Revision, code 065.4) also increased because the code appears quite infrequently, making statistical inferences unreliable. We also attempted to use deaths as a marker for disease severity, but we could not detect an increase in disease severity in our analysis because number of deaths was insufficient to accurately estimate a mortality rate.

Dengue and dengue hemorrhagic fever have been described as potential public health threats for residents of the US mainland (*14*). Despite the proximity of circulating dengue virus to the continental United States and the spread of the vector mosquitoes (*Aedes aegypti* and *Ae. albopictus*) to at least 26 states (*15*), autochthonous cases in the continental United States have been relatively rare (*5*) until the recent Florida outbreak. The increase in reported cases that we have documented highlights a potential risk for dengue spread within the United States. Although dengue fever was previously classified as reportable in some states, it did not become a reportable illness at the national level until 2010. Thus, some time is required before cases reported to public health departments can be used to establish reliable statistical estimates of national trends. Furthermore, the number of cases may not be linked to other relevant clinical data.

The major limitation to our study is that we used administrative data, and thus we did not have access to laboratory data or patients' travel histories. In addition, milder cases treated on an outpatient basis were not captured. Nevertheless, our results indicate that the decision to make dengue fever a reportable disease in the United States was warranted and that increased vigilance focused on these new surveillance data is needed. In addition, administrative data, as we describe here, can be used to estimate the effects and severity of illness attributable to dengue.

Dr Streit is an assistant professor of medicine at the University of Iowa where she an infectious disease specialist and the director of the travel medicine clinic. Her research interests include tropical medicine and related phenomena.

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Detection and Phylogenetic Characterization of Human Hepatitis E Virus Strains, Czech Republic

Petra Vasickova, Michal Slany, Pavel Chalupa, Michal Holub, Radek Svoboda, and Ivo Pavlik

To determine the origin of hepatitis E virus in the Czech Republic, we analyzed patient clinical samples. Five isolates of genotypes 3e, 3f, and 3g were obtained. Their genetic relatedness with Czech strains from domestic pigs and wild boars and patient recollections suggest an autochthonous source likely linked to consumption of contaminated pork.

Hepatitis E virus (HEV) is a leading cause of epidemics and sporadic cases of enterically transmitted hepatitis worldwide. The zoonotic potential of HEV was recognized recently, and pigs and other animal species were considered natural reservoirs for the virus (*1*). Currently, mammalian HEV strains segregate into 4 major genotypes. The relative conservation of genotypes 1 and 2 corresponds to their primary circulation within humans. Genotype 1 consists of epidemic strains from developing countries in Asia and Africa, and representatives of genotype 2 have been described in Mexico and African countries. The diversity of genotypes 3 and 4 is related to their origin from a variety of animal species. Genotype 3 is widely distributed and has been isolated from patients with sporadic cases of acute hepatitis E worldwide. Genotype 4 contains strains of human and animal origin, especially in isolates from Asian countries (*2*,*3*).

In the Czech Republic, hepatitis E incidence has been increasing since the first case was described in 1996. From 1996 through 2005, a total of 159 cases of HEV infection were reported; 23% of those cases were associated with travel to industrialized countries (*4*). In 2005, 37 hepatitis E patients were reported in the Czech Republic, while in 2009 the number increased to 99 (*5*). On the basis of these data, extensive genomic variability among HEV isolates and their known geographic distribution, we conducted a phylogenetic analysis of HEV isolates from clinical samples of Czech patients with acute hepatitis E to determine the origin of the infection.

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The Study

Stool samples from a total of 10 patients with serologically confirmed acute hepatitis E were tested (online Appendix Table, www.cdc.gov/EID/content/17/5/917-appT.htm). Informed consent was obtained from all patients involved in this study (Ethics Committee, University Hospital Bulovka; IRB00002721).

Two hundred and fifty milligrams of stool sample was suspended in 2.25 mL of phosphate-buffered saline, homogenized by vortexing, and clarified by centrifugation at 3,000× *g* for 15 min. RNA was extracted from 140 μL of supernatant by using the QIAamp Viral RNA Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. A positive template control was isolated from swine feces positive for HEV RNA from a previous study (*6*), and a water sample was included as a negative control.

Detection of HEV RNA was performed by nested reverse transcription-PCR with 2 sets of degenerate primers that targeted open reading frame 1 (ORF1) and the overlapping part of ORF2 and ORF3 (ORF2/3) of the HEV genome as described previously (*6*). Nondiluted and 10-fold diluted samples of isolated RNA were analyzed. Subsequently, the specific PCR product (length 242 bp, ORF1 primers) obtained from 2 independent RNA isolations was prepared for sequencing by the QIAquick PCR Purification Kit (QIAGEN). Both strands were sequenced at Eurofins MWG Operon (Ebersberg, Germany).

The sequencing and phylogenetic analysis were carried out by using MEGA version 3.1 software (www.megasoftware.net). The method of neighbor-joining with 1,000 replications in the bootstrap test was used for phylogenetic analysis (*7*), and bootstrap values >50% were considered significant. The 5 most similar HEV sequences available in GenBank database were chosen for each presented Czech human HEV isolate (isolate CZhHEV), according to the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Selected sequences were supplemented with representatives of genotype 3e (strain G2, GenBank accession no. AF110389), 3f (strain G1, accession no. AF110388), and 3g (strain Osh 205, accession no. AF455784) (*2*).

HEV RNA was detected in clinical samples from 6 of 10 patients whose samples were tested. Results obtained by using primers specific for ORF1 and ORF2/3 of the HEV genome were in agreement, except for material

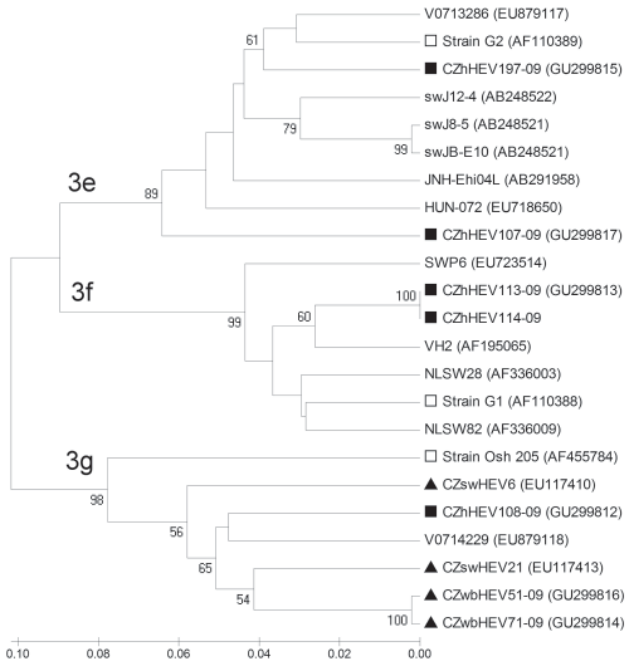

Figure. Phylogenetic tree constructed with MEGA version 3.1 software (www.megasoftware.net) by using the neighbor-joining method with 1,000 replication in bootstrap test based on 242-bp–long sequences within open reading frame 1 (OFR1) of hepatitis E virus (HEV) isolates and only bootstrap values (percentages) >50 are indicated on the tree. Key: ■, sequences originating from 5 Czech patients; □, representatives of genotype 3 subtypes: 3e (strain G2), 3f (strain G1), and 3g (strain Osh 205); and the 5 HEV strains most similar to human isolates from the Czech Republic: human strains from Germany, V0713286 and V0714229; swine strains from Japan, swJ12–4, swJ8–5, and swJB-E10; human strain from Japan, JNH-Ehi04L; swine strain from Hungary, HUN-072; swine strains from the Netherlands, NLSW28 and NLSW82; swine strain from Spain, SWP6; human strain from Spain, VH2; ▲, strains from the Czech Republic originating from domestic pigs, CZswHEV6 and CZswHEV21; and from wild boars, CZwbHEV51–09 and CZwbHEV71–09. GenBank accession numbers of chosen sequences are included in the phylogenetic tree. Scale bar indicates nucleotide substitutions per site.

from 1 patient. Specific PCR products of 5 CZhHEV isolates were sequenced (online Appendix Table). The sequence analysis showed that sequences of CZhHEV isolates shared a homology of 81.4% (CZhHEV107-09 and CZhHEV113-09) to 100.0% (CZhHEV113-09 and CZhHEV114-09).

Phylogenetic analysis showed that isolates CZhHEV107-09 and CZhHEV197-09 were most genetically similar to subtype 3e (strain G2) and were clustered together with sequences from a pig from Hungary (HUN-072, accession no. EU718650), a person from Germany (V0713286, accession no. EU7879117), pigs from Japan (swJ8–5, accession no. AB248521; swJ12–4, accession no. AB248522; swJB-E10, accession no. AB481226), and a patient from Japan (JNH-Ehi04L, accession no. AB291958). Isolate CZhHEV107-09 had highest sequence homology (88.8%) with strain HUN-072, while isolate CZhHEV197-09 and Greek strain G2 shared homology of 93.0%.

Isolate CZhHEV108-09 belonged to the subtype 3g, together with other Czech strains from wild boars (CZwb51-09, accession no. GU299814; CZwbHEV71-09, accession no. GU299816), Czech domestic pigs (CZswHEV21, accession no. EU117413; CZswHEV6, accession no. EU117410), German strain V0714229 (GenBank accession no. EU879118) and strain Osh205. CZhHEV108-09 shared highest homology (91.3%) with strain CZwbHEV51-09 and strain V0714229.

Identical sequences of CZhHEV113-09 and CZhHEV114-09 isolates were clustered to subtype 3f (strain G1). A human strain from Spain (VH2, accession no. AF195065), swine strains from the Netherlands (NLSW28, accession no. AF336003; NLSW82, accession no. AF336009) and from Spain (SWP6; accession no. EU723514) also belonged in subtype 3f. The highest sequence homology (95.0%) was found for isolates CZhHEV113-09, CZhHEV114-09, and strain VH2 (Figure). Sequences of isolates CZhHEV107-09, CZhHEV108–09, CZhHEV113-09, and CZhHEV197-09 have been deposited in GenBank under accession nos. GU299817, GU299812, GU299813, and GU299815, respectively.

Conclusions

We tested stool samples of 10 patients with serologically confirmed acute hepatitis E and, despite this confirmation, have detected HEV RNA in only 6 of these patients (online Appendix Table; Figure). Most serologic assays for diagnosing hepatitis E use recombinant proteins of genotypes 1 and 2, and these tests may be less sensitive and specific for detection of genotypes 3 and 4 of genus *Hepevirus* (*8*,*9*). These results were confirmed by a study performed in the Czech Republic, which showed that 28 (47.5%) of 59 IgM anti-HEV positive cases were in fact false positives (unpub. data).

According to Lu et al. (*2*), subtypes 3c, 3e, 3f, 3h, and 3i have been mainly identified in Europe and subtype 3g in Asia. In our study, isolates CZhHEV107-09 and CZhHEV197-09 were genetically related to subtype 3e, while identical CZhHEV113-09 and CZhHEV114-09 belonged to subtype 3f. Isolate CZhHEV108-09 clustered with subtype 3g and a strain from a wild boar from the Czech Republic (CZwbHEV51-09) and a strain from a human in Germany that was associated with consumption of wild boar meat, offal, and locally produced meat products (*10*). These findings supported the idea of zoonotic transmission of HEV. Moreover, other Czech strains from domestic pigs belonged also in subtypes 3g and 3f (Figure).

The patient from whose stool isolate CZhHEV108-09 was identified reported eating homemade pig-slaughter products 1 month before the first symptoms of hepatitis E and also visiting sushi bars in Germany that served grilled pork. As Lupulovic et al. (*11*) reported, the prevalence of anti-HEV immunoglobulin (Ig) G in pigs raised in family backyards is similar to the prevalence in those bred on commercial farms. Thus, infection obtained during home slaughter is probable. On the other hand, Germany is one of the biggest exporters of domestic pigs and pork meat into the Czech Republic. Based on geographic closeness, circulation of subtype 3g between the 2 countries is possible. Moreover, that HEV infection resulted from the consumption of insufficiently heat-treated meat in sushi bars cannot be excluded.

The identity of sequences of isolates CZhHEV113-09 and CZhHEV114-09 has strongly hinted at an identical source of HEV. Both patients lived in the same city and had overlapping times of hospitalization. Furthermore, the patients reported consumption of pork and that they used the same knife and chopping board for raw and cooked meat (online Appendix Table). Therefore, cross-contamination during the meat processing is probable.

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Genetic Characterization of West Nile Virus Lineage 2, Greece, 2010

Anna Papa, Tamás Bakonyi, Kyriaki Xanthopoulou, Ana Vázquez, Antonio Tenorio, and Norbert Nowotny

We conducted a complete genome analysis of a West Nile virus detected in *Culex pipiens* mosquitoes during a severe outbreak of human West Nile disease in Greece 2010. The virus showed closest genetic relationship to the lineage 2 strain that emerged in Hungary in 2004; increased virulence may be associated with amino acid substitution H249P.

West Nile virus (WNV) is a flavivirus maintained in an enzootic cycle between bird amplifying hosts and ornithophilic mosquito vectors, mainly *Culex* species; humans, horses, and other mammals are incidental hosts. Although most human WNV infections remain subclinical, febrile illness develops in ≈20% of infected persons and neuroinvasive disease in <1%. Severe disease is more frequent among the elderly and immunocompromised (*1*).

WNV strains are classified into at least 7 putative genetic lineages (*2*). Lineage 1 strains are the most widespread, found in Africa, Europe, Asia, Australasia ("Kunjin virus"), and America, while lineage 2 strains are mainly distributed in sub-Saharan Africa and Madagascar. WNV of proposed lineage 3 ("Rabensburg virus") is circulating in certain *Culex* and *Aedes* species mosquitoes in southern Moravia, Czech Republic, close to the Austrian border, without recognized pathogenicity for mammals (*3*). Strain LEIV-Krns88-190, isolated from *Dermacentor marginatus* ticks from the Caucasus represents proposed lineage 4 of WNV. A new lineage, lineage 5, has been proposed for Indian isolates previously comprising lineage 1c, and a reclassification as lineage 6 has been proposed for the Sarawak Kunjin virus strain, which is markedly different from the other Kunjin viruses. Furthermore, a seventh lineage has been suggested for the African Koutango virus, which is closely related to WNV (*2*), and an eighth lineage has been proposed on the basis of WNV sequences detected in *Cx. pipiens* mosquitoes captured in southern Spain in summer 2006 (*4*).

WNV strains differ considerably in virulence and neuroinvasiveness. Since neuroinvasive isolates mainly belonged to lineage 1, lineage 2 strains were previously considered to be less virulent. Recent studies, however, indicate that several highly virulent and neuroinvasive strains of lineage 2 WNV were detected in southern Africa (*5*).

Until 2004, only lineage 1 and 3 WNV strains had been found in Europe. A lineage 2 strain emerged in 2004 in Hungary in birds of prey (*6*), which established itself in the region and exhibited explosive geographic spread in 2008 throughout Hungary and into eastern Austria. Besides deaths in birds and domesticated mammals, human neurologic WNV cases have been diagnosed in the affected regions during the epidemic seasons since 2004. However, the human cases of WNV neuroinvasive disease have been comparatively rare and rather mild with no deaths.

In Greece, where WNV cases had not previously been reported, a 2007 study, conducted in an area near a delta where 4 rivers enter the Aegean Sea, 4 (1%) of 392 persons exhibited neutralizing WNV antibodies, 2 with high titers. These findings suggested that WNV, or an antigenically closely related flavivirus, circulates, at least locally, in rural areas in Greece (*7*). This area was the focus of a large WNV outbreak in summer–autumn 2010 (*8*). Up to October 4, 2010, a total of 191 neuroinvasive human cases have been laboratory diagnosed, with 32 deaths, all in elderly patients.

Soon after the first human cases were recognized, mosquitoes were trapped at the sites where the cases occurred and tested for WNV. One pool, consisting of 50 *Cx. pipiens* mosquitoes, trapped during the night of August 10, 2010, in Nea Santa (40.84194°N, 22.91499°E), a village 30 km northeast of Thessaloniki, was found to be positive for WNV RNA by reverse transcription–PCR. Sequencing of a 146-nt fragment of the nonstructural protein 5 (NS5) gene gave the first evidence that the virus belongs to lineage 2, and that it is highly similar to the strain that emerged in Hungary in 2004 and to isolates from fatal human and animal cases in South Africa (*9*). The purpose of this study was to establish the complete genomic sequence of the WNV identified in a pool of mosquitoes trapped at the site of an ongoing West Nile disease epidemic in humans, and investigate it for potential (neuro)virulence/pathogenicity markers.

The Study

To amplify overlapping PCR products of the WNV from the Greek outbreak, we used RNA from the above-mentioned mosquito pool and reverse transcription–PCRs designed for the amplification of the complete genome of lineage 2 WNV, which had emerged in Hungary in 2004

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(strain goshawk-Hungary-2004, GenBank accession no. DQ116961; *6*). Amplification products were directly sequenced in both directions; sequences were aligned and compiled, and an 11,028-nt, continuous sequence was obtained, representing the nearly complete genome sequence of the virus, designated Nea Santa-Greece-2010. Because of the limited amount of sample material, the last 20 nt of the 5′ and 3′ ends were not determined by rapid amplification of cDNA ends. The nucleotide sequence was deposited in GenBank database under accession no. HQ537483.

The nucleotide sequence of the putative open reading frame 1 (starting at nt position 97) was translated to a 3,434-aa polypeptide sequence. The nucleotide and putative amino acid sequences were aligned with all complete lineage 2 WNV nt and aa sequences available in GenBank, as well as with representatives of other WNV lineages.

The highest (99.6%) nucleotide identity of the Nea Santa-Greece-2010 sequence was found to the goshawk-Hungary-2004 strain. Only single nucleotide substitutions (n = 44) were detected, equally distributed over the genome. The putative aa sequences of the polyprotein precursor of the 2 viruses were 99.7% identical. The alignment of the putative precursor polypeptide sequences showed aa substitutions unique for the European lineage 2 WNVs. These substitutions were found in the nonstructural proteins (Table).

Several studies have focused on the determination of genetic markers for pathogenicity and virulence of WNV strains. In the New York strain of WNV, envelope protein glycosylation proved to be a molecular determinant of neuroinvasiveness (*10*). The $A_{30}P$ substitution in the NS2A protein reduced the ability of the virus to inhibit interferon induction (*11*). The introduction of a $T_{249}P$ amino acid substitution in the NS3 helicase in a low-virulence strain was sufficient to generate a phenotype highly virulent to American crows (*12*). The $C_{102}S$ mutation in the NS4A protein also influenced virulence and thermosensitivity in a mouse model (*13*). An adaptive mutation $E_{249}G$ in the same protein reduced virus replication in mouse cells (*14*). In lineage 2 strains, substitutions in the NS3 protein ($S_{160}A$ and $R_{298}G$), NS4A protein ($A_{79}T$), and NS5 protein ($T_{614}P$, $M_{625}R$, and $M_{626}R$) were predicted to be possible virulence markers (*15*). All these foci were checked in the Nea Santa-Greece-2010 strain, but differences were not found, except for the 249 residue of the NS3 protein. All investigated lineage 2 viruses (including goshawk-Hungary-2004) contain histidine at this locus, while the Greek sequence contains proline, similar to several neuroinvasive lineage-1 WNV strains. Previous experimental studies have attributed this mutation to a higher capacity of the virus to replicate in corvids (*12*), which likely would result in increased virus transmission rates.

Viruses of lineages 3, 4, and 5 contain aspargine, threonine, and threonine at this locus, respectively (Table). The inferred phylogenetic relationships between the investigated complete genome nucleotide sequences are delineated in a phylogram (Figure). The Nea Santa-Greece-2010 and goshawk-Hungary-2004 viruses form a close, monophyletic group that clusters with neurovirulent lineage 2 strains isolated in South Africa.

Conclusions

Epidemiologic observations of the lineage 2 WNV in Europe during 2004–2010, and the close genetic relatedness of the WNV circulating in Hungary and eastern Austria to the virus identified in *Cx. pipiens* mosquitoes during the 2010 outbreak in Greece, indicate that, most likely, descendants of the goshawk-Hungary-2004 strain spread southward to the Balkan Peninsula and reached northern Greece. Our data indicate that an independent introduction of a highly similar lineage 2 WNV from Africa to northern Greece is unlikely. The importance of the $H_{249}P$ aa change in the NS3 protein of the Nea Santa-Greece-2010 virus should be verified in experimental studies. This aa change also emphasizes the value of close genetic monitoring of strains involved in additional European WNV outbreaks.

Table. Unique amino acid substitutions in the putative nonstructural proteins of the Nea Santa-Greece-2010 lineage 2 West Nile virus*

	Protein, aa position										
	NS1	NS2B		NS3		NS4B					NS5
Strain	44	88	119	11	249	14	23	32	49	113	190
Gr-10 (lin. 2)	Arg	Ile	Ile	Arg	**Pro**	Gly	Thr	Asn	Ala	Met	Arg
Hu-04 (lin. 2)	Arg	Ile	Val	Arg	His	Ser	Thr	Asn	Thr	Val	Lys
Other lin. 2 WNV strains	Lys	Met	Val	Lys	His	Ser†	Ala	Ser	Thr‡	Val	Lys
NY-99 (lin. 1)	Lys	Met	Val	Lys	**Pro**	Ser	Val	Glu	Thr	Val	Lys
Rab-97 (lin. 3)	Arg	Ile	Val	Lys	Asn	Ser	Thr	Asp	Ser	Val	Lys
Rus-98 (lin. 4)	Lys	Met	Val	Arg	Thr	Gly	Ser	Ser	Ser	Val	Lys
Ind-80 (lin. 5)	Lys	Met	Val	Lys	Thr	Gly	Ala	Glu	Thr	Val	Lys

*NS, nonstructural protein; aa, amino acid; lin., lineage; Arg, arginine; Ile, isoleucine; Pro, proline; Gly, glycine; Thr, threonine; Asn, asparagine; Ala, alanine; Met, methionine; Val, valine; His, histidine; Lys, lysine; Ser, serine; WNV, West Nile virus. Abbreviations of strains are explained in the Figure legend. **Boldface** indicates the His→Pro substitution at aa position 249.
†Except SE-90, WNFCG, Mad-78, and Sarafend (Gly).
‡Except Mad-78 (Ala).

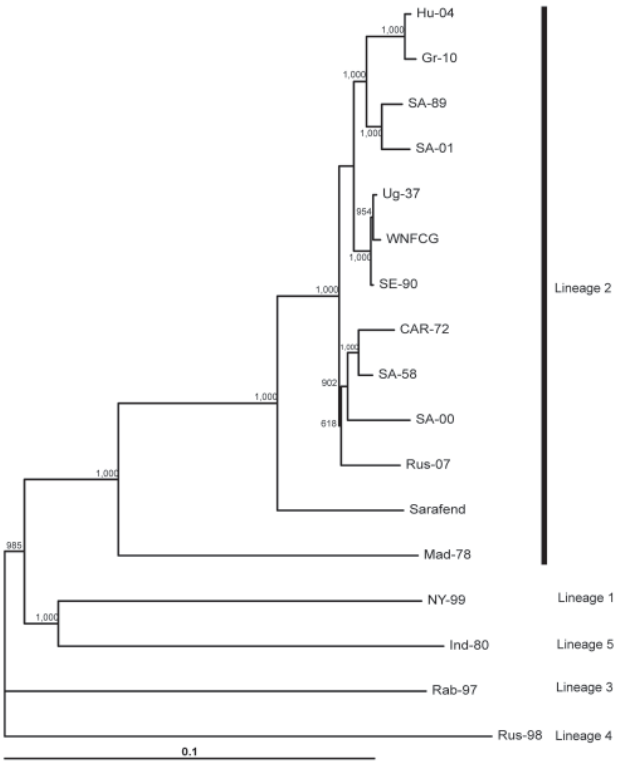

Figure. Neighbor-joining phylogram based on complete genome nucleotide sequences of selected West Nile virus strains. Strain abbreviations (isolation source, country, year, GenBank accession no.): Hu-04: *Accipiter gentilis*, Hungary, 2004, DQ116961; Gr-10: *Culex pipiens*, Greece, 2010, HQ537483; SA-89: human, South Africa, 1989, EF429197; SA-01: human, South Africa, 2001, EF429198; Ug-37: human, Uganda, 1937, AY532665; WNFCG: derivate of Ug-37, M12294; SE-90: *Mimomyia lacustris*, Senegal, 1990, DQ318019; CAR-72: *Cx. tigripes*, Central African Republic, 1972, DQ318020; SA-58: human, South Africa, 1958, EF429200; SA-00: human, South Africa, 2000, EF429199; Rus-07: human, Russia, 2007, FJ425721; Sarafend: derivate of Ug-37, AY688948; Mad-78: *Coracopsis vasa*, Madagascar, 1978, DQ176636; NY-99: human, USA, 1999, AF202541; Ind-80: human, India, 1980, DQ256476; Rab-97: *Cx. pipiens*, Czech Republic, 1997, AY765264; Rus-98: *Dermacentor marginatus*, Russia, 1998, AY277251. Rus-98 was used as outgroup. Bootstrap values of 1,000 replicates are shown. The main genetic lineages are indicated on the right. Horizontal bar shows genetic distance.

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The Crab Hole Mosquito Blues

Karl M. Johnson, Douglas F. Antczak, William H. Dietz, David H. Martin, and Thomas E. Walton

Venezuelan equine encephalomyelitis (VEE) epizoodemics were reported at 6–10-year intervals in northern South America beginning in the 1920s. In 1937, epizootic VEE virus was isolated from infected horse brain and shown as distinct from the North American equine encephalomyelitis viruses. Subsequently, epizootic and sylvatic strains were isolated in distinct ecosystems; isolates were characterized serologically as epizootic subtype I, variants A/B and C; or sylvatic (enzootic) subtype I, variants D, E, and F, and subtypes II, III, and IV. In 1969, variant I-A/B virus was transported from a major outbreak in northern South America to the borders of El Salvador, Guatemala, and Honduras. This musical poem describes the history and ecology of VEE viruses and the epidemiology of an unprecedented 1969 movement of VEE viruses from South America to equids and humans in Central America from Costa Rica to Guatemala and Belize and in Mexico and the United States that continued until 1972.

Crab Hole Mosquito[A] Blues

Written and performed by the MARU Health Angels Band[B]

Refrain: Mosquito's in his[C] crab hole, bidin' his time,
Venezuela virus working up the line,
Boys in Beltsville[D] heard the news,
Horses in Texas[E] got the crab hole blues.
Down in Maracay back in '36,
Kubes and Rios[F] found a virus doin' tricks,
Horses die, this one's gotta be,
New cause of 'cephalitis, V-E-E.[G]
Voice-over: Horses, mules, and donkeys are all susceptible.
The years roll by, the virus makes a score,
In Vene, Colombo, and Ecuador,[H]
Comes rain to the desert instead of dew,
And, VEE burns the coast of Peru.[I]
In Trinidad, Panama, they say "Hey, Hey,"
We got this creature like every day,
His swampy home, you can always tell,
By finding some rats and the *Culex* (*Mel*).[J]"
Voice-over: Mosquito, that is.

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Back at MARU, raggin' the brains,
We found that the virus had different strains
Horses convulse with the southern kind,
Northern virus leaves them feelin' fine.[K]"
Refrain
'Cause virus hits man by aerosol,
Could be used to cripple us all,
In Maryland, they grew it in vats,
Tested it out in monkeys and rats.[L]
The other Team outside the fence,
Lookin' for the answer that made some sense,
Got a strain from an old donkey,
Made a vaccine, TC-83.[M]
The offensive team tried to make some noise,
A cloud for the Commies, but not our boys,[N]
Did not work, that is why,
Nixon[O] said 'declassify'.
Voice-over: The Pentagon papers, volume 25, page 324, 1970… the New York Times, Los Angeles Times, the Washington Post, and the Christian Science Monitor….
Sleepy defense gets a Latin call,
New outbreak and it ain't small,
Took the vaccine off the wall,
It really worked to save them all.[P]
Refrain
Down in Guatemala, year of '69,
Horses started dying on the borderline,
Virus come down from Ecuador,
Nobody knows the reservoir.[Q]
Voice-over: But Mackenzie[R] has an idea.
Virus hopped the mountains, got to the sea,[S]
Vaccine brought in and given out free,
Virus kept movin', had to go,
All the way from here to Mexico.[T]
Cause everybody said, "Gotta stop that bug,
Gotta use the vaccine because there ain't no drug,"
Beltsville brethren said "Gotta wait,"
Afraid that the vaccine was MF-8.[U]
Voice-over: That means reversion to virulence.
Results today are plain to see,
Story's hot on the wire of Associated P,[V]
A million horses all over the West,
Are living proof the vaccine was best.[W]
Refrain
Voice-over: Addresses for reprints may be mailed to Karl M. Johnson.

A) The presentation is a chronologically, scientifically, and factually correct poetic and musical history of Venezuelan equine encephalomyelitis (VEE) viruses and epidemics through 1971. The music is a traditional US jug band song, a type of music—bluegrass—popular in rural areas of the eastern United States. Using a background of a guitar and everyday musical, rhythm, and percussion devices, such as whistling, blowing air across the mouth of a 1-gallon glass jug, scraping of a scrub brush on a metal washboard, humming harmonically into a kazoo, and the "hambone" (a rhythmic slapping of hands on arms, hands, and legs), the voices and lyrics convey a musical story to the audience.

The title reflects the discovery that *Deinocerites pseudes*, the crab hole mosquito, found along the Pacific coast of Central America was a competent vector of the epidemic and epizootic VEE virus that was causing disease and death in equids and humans at the time (*1*). The poetic dialogue was written and set to music in 1971 by scientists at the Middle America Research Unit (MARU), US Department of Health, Education, and Welfare (now Health and Human Services), National Institutes of Health, National Institute of Allergy and Infectious Diseases, US Public Health Service, and located in Ancon, Panama Canal Zone, during a major western hemispheric outbreak of VEE. MARU scientists had a >15-year history of VEE epidemiologic and virologic studies throughout Latin America to characterize the antigenic relationships between South American VEE virus strains of subtypes (I-A/B and I-C) that caused epizoodemics of equine and associated human disease and the Caribbean, Central American, Floridian, Mexican, Panamanian, and South American VEE virus strains of subtypes (I-D, I-E, I-F, II, III-A, III-B, and IV) that existed in sylvatic, enzootic cycles in the absence of equine disease but with occasional human infections and disease (*2*). In 1969, the transfer of equine virulent VEE virus from a raging epizoodemic in Colombia, Ecuador, and Peru to the frontier area of El Salvador, Guatemala, and Honduras precipitated a human and veterinary medical crisis in Central America, Mexico, and the United States that lasted until 1972 (*3*).

The Crab Hole Mosquito Blues was written as a scientific presentation for the international Workshop-Symposium on Venezuelan Encephalitis Virus sponsored by the Pan American Sanitary Bureau, Pan American Health Organization, World Health Organization, in Washington, DC, September 14–17, 1971. Although the lyrics of the Crab Hole Music Blues were not published in the Proceedings, other presentations from that meeting are documented (*4*).

B) Douglas F. Antczak, vocals, guitar, kazoo; William H. Dietz, vocals, jug, kazoo; Karl M. Johnson, vocals, kazoo; David H. Martin, kazoo, recording engineer; and Thomas E. Walton, washboard and scrub brush, hambone, kazoo, vocals.

C) Gender-specific error. Only female mosquitoes (and females of other hematophagous insects, e.g., culicoids, phlebotomids) take a blood meal, which is needed to provide protein necessary for ovulation; only female mosquitoes are infected with and transmit VEE viruses (and other mosquito-borne arboviruses). Male mosquitoes feed on plant source liquids and water.

D) Beltsville, Maryland, USA, the headquarters at that time of the regulatory officials in the US Department of Agriculture (USDA) responsible for the decision to recommend application of preventive vaccines to the Secretary of Agriculture in the face of an epizootic. Hesitation to vaccinate was predicated on lack of evidence that reversion of virulence of the attenuated vaccine virus could not occur, international trade considerations, international practices and agreements, and authorities and responsibilities delegated only to the Secretary of Agriculture.

E) From 1969, the epizoodemic moved southeast, eventually reaching northwestern Costa Rica in August 1970, and northward, eventually reaching Texas in late June 1971 (*4*).

F) The Venezuelan agricultural and veterinary research laboratories are located in Maracay, Aragua State, Venezuela. Kubes and Rios first isolated, identified, and named VEE virus, then sent the isolate to the United States for confirmation that the South American virus was antigenically distinct from the North American eastern and western equine encephalomyelitis viruses (*5*,*6*).

G) The antigenically related and clinically similar eastern and western equine encephalomyelitis viruses had been isolated and identified early in the 1930s in the United States (*7*,*8*).

H) Periodic outbreaks of VEE had occurred in Colombia, Ecuador, Peru, and Venezuela since at least the 1920s, with hundreds of thousands of equine illnesses and tens of thousands of deaths; equids are the primary virus amplifier hosts for human infections (*9*,*10*).

I) Throughout the history of VEE in northern South America, periodic epizoodemics in tropical dry and tropical thorn forests were often associated with unseasonably heavy rainfall and flooding during the normal dry seasons; during interepizootic periods, epizoodemic virus could not be isolated. The great Atacama Desert stretches along the Pacific coast from northern Chile and along coastal Peru nearly to the border with Ecuador; rare, but occasional, rainfall interrupts the barrenness of this parched, hostile environment permitting infrequent but noteworthy incursions of mosquitoes and epizoodemic VEE virus (*4*).

J) In contrast, in swampy or jungle areas (tropical wet forest) where a definable dry season does not occur normally in countries of Central America and eastern South America and in Panama, Mexico, the Florida Everglades, and several Caribbean islands, field studies by scientists at MARU (*11*), the Center for Disease Control (now Centers for Disease Control and Prevention, Atlanta, GA, USA) (*12*), the Trinidad Virus Research Laboratory (Port of Spain) (*13*), Rockefeller Foundation Laboratory (Belem, Brazil) (*14*), and the Gorgas Memorial Laboratory (Panama City, Panama) (*15*) had demonstrated presence of antigenically related VEE virus strains; resident equids in these sylvatic foci had antibody without signs of disease, but incursions by humans into these sylvatic or endemic areas often resulted in infections and disease. Sylvatic cycles were found in swampy areas in which floating species of water lettuce, *Pistia stratiotes*, provided appropriate habitat for mosquito species of the subgenus *Culex* (*Melanoconion*), the vectors of sylvatic subtypes and variants of VEE virus. The epidemiologic cycle involves sylvatic virus transmission by species of terrestrial rodents and possibly birds and arboreal rodents (*4*).

K) Sylvatic virus subtypes were of low or no virulence to experimentally infected equids. The virulence of epizootic subtypes was high, with fatality rates to >90% of infected equids (*16*).

L) Historically, VEE virus has been a pathogen studied for aerosol release as a potential biologic weapon. In the United States at the Army Research and Development Command, (now the Army Medical Research Institute for Infectious Diseases, USAMRIID), Fort Detrick, MD, the former Soviet Union and perhaps, elsewhere, VEE virus was studied as a possible offensive weapon. (For more information about the history of the US biologic warfare program and Fort Detrick, go to the following websites: www.detrick.army.mil/cutting_edge/index.cfm, www7.nationalacademies.org/archives/cbw.html, and www.bordeninstitute.army.mil/published.html.)

M) In the defensive research programs at USAMRIID, an equine-virulent isolate from a donkey was serially passed in fetal guinea pig heart cell cultures to produce an attenuated vaccine, strain TC-83, for use in at-risk laboratory and military personnel (*4*). Attenuated strain TC-83 was derived from the Trinidad donkey number 1 isolate from a diseased donkey in that country during a 1940s epizootic that spilled over to Trinidad and Tobago from mainland Venezuela (*17*).

N) "Commies" [communists] reflected Cold War–era concerns about military personnel of the former Union of Soviet Socialist Republics and their allies. Aerosol releases of biological, chemical, and radioactive weapons are notoriously difficult to control, leading to use of vaccine, if available, and other, more cumbersome measures to protect military personnel.

O) Recognizing the difficulties in controlling and using biological weapons, unauthorized release of classified documents, and the moral outrage of US citizens and world public opinion against biological weapons, President Richard M. Nixon cancelled the offensive biological weapons development programs.

P) Beginning in 1967, a major epizoodemic of VEE occurred across northern South America (*4*). Requests to the US State Department and US military authorities resulted in release of attenuated VEE virus vaccine strain TC-83 for emergency use in equids to stop equine disease and interrupt human infections. Vaccine was effective, but the silent epizoodemic tongue of virus transmission repeatedly had moved ahead of the vaccination teams through populations of susceptible hosts and competent vectors.

Q) In an unprecedented biomedical event in the history of VEE, the epizoodemic VEE virus subtype was transported from northern South America to Central America (*3*). Empirical and circumstantial evidence, such as discovery by scientific investigators of empty vials of VEE virus vaccine labeled by a manufacturer in South America, suggested that a formalin-inactivated VEE virus vaccine was imported to vaccinate valuable horses at breeding farms in Guatemala by worried ranch owners (K.M. Johnson, unpub. data). VEE virus, like poliovirus and other viruses, is notoriously difficult to inactivate. Safety tests of such inactivated VEE virus vaccines in laboratory systems, e.g., cell cultures and laboratory animals, are exquisitely less sensitive than susceptible equids to residual active virus. Non–inactivated virus has been postulated to replicate in equids to high titers and to be infectious for the local populations of competent and capable mosquito vectors.

The reservoir of epizoodemic VEE virus during interepizootic periods is unknown, but studies during the 1990s and 2000s suggest an enhanced virulence of certain isolates of sylvatic virus subtypes and strains for equids, which occasionally are replicated to high titers under undetermined favorable conditions, with subsequent selection of an epizoodemic clone from a mixed virus population that causes clinical VEE and infects mosquitoes.

R) An internationally recognized clinical and field research expert on zoonotic diseases, Ronald B. Mackenzie was a visionary and astute medical scientist with The Rockefeller Foundation in Cali, Colombia.

S) VEE virus was transported by mosquitoes and possibly through transportation of asymptomatic but infected horses from the disease or danger zones to unaffected zones where susceptible equids were subsequently infected. Disease was documented along the

Pacific and Caribbean coasts in the tropical dry and thorn forest environments that have been the traditional cattle-raising areas of Central and South America and in Mexico, where tropical wet forest and swampy environments that support sylvatic VEE viruses are irregularly located and noncontiguous or do not occur.

T) Because of the lack of understanding of the epidemiologic cycle and virus-vector incubation requirements, the epizoodemic wave of infected mosquitoes and equids incubating the virus had not been anticipated to precede vaccination teams routinely into new areas of susceptible equids by 2–3 weeks. The disease moved to the southeast along the Pacific coast to northwestern Costa Rica, where the advance finally was stopped, protecting Panama, probably because of a combination of vaccination, presence of larger sylvatic foci in which larger numbers of resident equids were already immune, and a belt of lowland and montane rain forests where there are fewer cattle and horses and that stretches along the Pacific coast of southwestern Costa Rica to northwestern Panama; antibody to sylvatic virus strains provides cross-protection against epizoodemic virus strains. The virus crossed the Isthmus of Tehuantepec in Mexico and moved up both the Caribbean and Pacific coasts, finally reaching Texas in late June 1971 (detection of VEE in Texas before July 4 was predicted months earlier by K.M. Johnson). Despite thorough vaccination and aerial application of insecticides, some disease activity persisted in Mexico until 1972. The last activity from the epizootic in the Western Hemisphere occurred in 1973.

U) MF-8 is an isolate of the epizoodemic VEE virus subtype I-A/B from Honduras isolated by Miguel Figueroa, a Honduran scientist working at MARU. Despite proven efficacy and safety of the attenuated VEE virus vaccine in South and Central America and Mexico, USDA authorities delayed application of the vaccine until the first cases were diagnosed in Texas. Official USDA emergency response policies and regulations at that time did not include the option to use vaccines to interdict threats of foreign animal diseases in the absence of documented disease within the United States (application of vaccines in the USDA Emergency Response Plan to incursions of foreign animal diseases was authorized in 2000). Because of bilateral and international agreements and policies regarding emergency disease responses, the adverse impact of applying foreign animal disease vaccines on the exportation of US livestock and agricultural products internationally and the politically charged decision to change existing policy, an authority delegated only to the US Secretary of Agriculture, vaccine was acquired and stockpiled along the Mexico–US border but could not be applied until VEE was diagnosed.

V) Associated P = the Associated Press news agency.

W) Attenuated VEE virus vaccine was safe, effective, and stable, and reversion to virulence did not occur (*18*,*19*). A potentially catastrophic disaster was marginalized and ultimately stopped in the Western Hemisphere by application of vaccine and other control techniques. Hundreds of thousands of equids and thousands of humans were saved by the emergency responses of veterinary and medical officials in every country from Colombia-Ecuador-Peru-Venezuela to Mexico and the United States; among the 13 at-risk nations, only Panama (and, in addition the Caribbean islands and other countries of South America) was spared from this crisis.

The successful application to veterinary use of a vaccine developed by the US military as a defensive tool for use in troops and at-risk laboratory personnel was an unforeseen and unanticipated benefit of the US Department of Defense research program. President Franklin D. Roosevelt authorized the US Army through a civilian agency to develop the US biological warfare program with offensive and defensive objectives in 1942. Laboratories and pilot plants were constructed at Camp (later Fort) Detrick, Maryland; the Special Procedures program from which the Special Immunizations Program evolved was one of the earliest operations to open. The Special Immunizations Program is responsible for the investigational vaccines, including strain TC-83, which were developed and are used under the Investigational New Drug authority and guidelines of the Food and Drug Administration. Seed stock of strain TC-83 virus was made available to the biologics industries of Western Hemisphere countries. Strain TC-83 and other next-generation iterations of the original vaccine, including an inactivated strain TC-83 product (C-84), are used or available in many countries of the Western Hemisphere.

Dr Johnson served as director of MARU during 1964–1975, established the Centers for Disease Control and Prevention maximum biocontainment laboratory in Atlanta, where he worked with hemorrhagic fever viruses, and later worked with hemorrhagic fever viruses at USAMRIID at Fort Detrick. His scientific interests include arthropod-borne viruses, hemorrhagic fever viruses, biological safety, and the design of BioSafety Level 4 biocontainment laboratories for research with highly pathogenic, human-lethal zoonotic viruses.

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Plasmodium vivax Seroprevalence in Bred Cynomolgus Monkeys, China

To the Editor: Malaria caused by *Plasmodium* spp. is one of the most prevalent parasitic diseases in the world, especially in tropical countries. *P. vivax* represents the second most prevalent of malaria species. Therefore, as measures to control the high death rates in humans caused by *P. falciparum* become more effective, *P. vivax* is likely to become the primary malaria threat (*1*). In *P. vivax* infection, the main clinical signs and symptoms are fever, chills, nausea and vomiting, generalized body pain and fatigue, toxic shock and pulmonary edema, retinal hemorrhage, renal failure, uremic encephalopathy, and thrombocytopenia (*2*–*6*).

Because of their phylogenetic proximity to humans, nonhuman primates have been used extensively as animal models of human diseases (*7*). Thousands of workers come in contact with monkeys, but little information about the prevalence of *P. vivax* in bred cynomolgus monkeys (*Macaca fascicularis*) is available worldwide. The objective of our investigation was to examine whether *P. vivax* seroprevalence is present in bred cynomolgus monkeys in the People's Republic of China.

A total of 328 blood samples were collected by venous puncture during June 2008–September 2009. Of these, 224 blood samples were from 4 nonhuman primate centers in Guangxi Zhuang Nationality Autonomous Region and 108 blood samples were from 2 nonhuman primate centers in Guangdong Province. All of the cynomolgus monkeys were caged. Each cage has 2 rooms, 1 indoors and 1 outdoors. The monkeys spend ≈10 hours in the outdoor room each day during the daytime. The age and sex of monkeys are listed in the Table. Serum samples were separated and stored at –20°C before testing.

Serum samples were tested for *P. vivax* antibodies by using a commercially available ELISA kit (Tiancheng Yiliu Co., Ltd, Shanghai, China), according to the manufacturer's instructions. This kit uses biotinylated anti–*P. vivax* as a coating antigen and is specifically for monkeys. Positive and negative control serum samples were provided in the kit and included in each test. Those samples with doubtful results were retested.

Differences in the seroprevalence of *P. vivax* in bred cynomolgus monkeys according to sex and area were analyzed by using the χ^2 test in SPSS 13.0 standard version for Windows (SPSS Inc., Chicago, IL, USA). The differences were considered to be statistically significant when the p value obtained was <0.05.

The total prevalence of anti–*P. vivax* antibodies in bred cynomolgus monkeys in southern China was 3.4% (11/328), which was lower than the prevalence of anti–*P. vivax* antibodies in captured monkeys (*Alouatta seniculus*, *Saguinus midas*, and *Pithecia pithecia*) in French Guiana (*8*). The prevalence in female monkeys (3.1%, 5/161) was slightly lower than that in male monkeys (3.6%, 6/167); the seroprevalence of 3.6% (8/224) in Guangxi Zhuang Nationality Autonomous Region was slightly higher than that in Guangdong Province (2.9%, 3/104) (Table), but these differences were not significant (p>0.05). A total of 52 monkey serum samples from nonhuman primate center E in Guangdong Province were found seronegative for *P. vivax* antibodies (Table). The difference in prevalence of *P. vivax* antibodies in different nonhuman primate centers may be related to differences in ecologic and geographic conditions, climate conditions, as well as in the management practices. All of the nonhuman primate centers are surrounded by hills or paddy fields, and the environment is favorable for *Anopheles* mosquitoes. The mosquito control measures, including the use of antimosquito insecticides and good drainage facilities for preventing water collection on the ground, were better executed at nonhuman primate center E than at the other nonhuman primate centers.

Table. Prevalence of *Plasmodium vivax* antibodies in serum samples, from bred cynomolgus monkeys in southern China, determined by ELISA, 2008–2009*

		Female			Male			Total	
Province	Primate center	Age, y	No. positive/ no. examined	Prevalence, %	Age, y	No. positive/ no. examined	Prevalence, %	No. positive/ no. examined	Prevalence, %
GX	A	3.0–5.0	0/20	0	3.0–6.5	1/30	3.3	1/50	2
	B	3.0–5.0	0/29	0	3.0–5.5	1/33	3.0	1/62	1.6
	C	3.0–5.0	1/27	3.7	3.0–5.5	2/33	6.1	3/60	5
	D	2.0–5.0	2/31	6.5	3.0–5.0	1/21	4.8	3/52	5.8
	Subtotal	2.0–5.0	3/107	2.8	3.0–6.5	5/117	4.3	8/224	3.6
GD	E	2.5–3.0	0/14	0	2.5–3.0	0/13	0	0/27	0
	E	7.0–8.0	0/12	0	8.0–9.0	0/13	0	0/25	0
	F	2.5–3.0	2/28	7.1	2.5–6.0	1/24	4.2	3/52	5.8
	Subtotal	2.5–8.0	2/54	3.7	2.5–9.0	1/50	2	3/104	2.9
Total		–	5/161	3.1	–	6/167	3.6	11/328	3.4

*GX, Guangxi Zhuang Nationality Autonomous Region; GD, Guangdong Province; –, not applicable.

Our survey showed *P. vivax* seropositivity in 5 of the 6 nonhuman primate centers in southern China, which is a potential health problem for bred cynomolgus monkeys. This finding also indicates the risk for infection with *P. vivax* for the employees of these nonhuman primate centers. Therefore, studies are warranted that assess the seroprevalence of *P. vivax* infection in persons who work in these nonhuman primate centers, as well as the seroprevalence of *P. vivax* infection in wild monkeys.

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Imported Dengue Virus Serotype 3, Yemen to Italy, 2010

To the Editor: Dengue is a mosquito-transmitted viral disease endemic to the tropics and subtropics worldwide. It is caused by 4 dengue virus serotypes (DENV-1–4) that belong to the genus *Flavivirus*. The disease varies from dengue fever to life-threatening hemorrhagic fever and shock that are associated with secondary infections. During recent decades, dengue incidence and prevalence have increased in disease-endemic areas, and the disease has been increasingly recognized in travelers (*1*). We report a case of dengue in a man who had traveled to Yemen.

In June 2010, a 38-year-old Italian man was admitted to the hospital for high fever (maximum 39.5°C) after a 1-week work-related stay in Yemen, near Mukalla, in the province of Hadhramaut. The patient had visited the countryside where he was heavily bitten by mosquitoes.

On the third day after onset of fever, the patient started to experience strong and unremitting frontal and retro-orbital headache and joint pains, which lasted for 5 days. He also experienced vomiting. Laboratory test results showed mild leukopenia (2.41 $\times 10^3$ cells/mm^3) and lowered platelet counts (96 $\times 10^3$ cells/mm^3), increased liver alanine aminotransferase levels (151 U/L), and mildly abnormal blood clotting (prothrombin time, international normalized ratio 1.24). In 1 week, the patient started to recover and was discharged from the hospital. The patient received antimicrobial (levoxacin) and antipyretic (acetaminophen) drugs. Laboratory testing after discharge showed increased levels of hepatic enzymes, which reached maximum levels on day 13 after onset of symptoms (alanine aminotransferase 669 U/L) and decreased to within reference limits in 1 month.

A plasma sample taken on day 6 after disease onset was positive for flavivirus RNA by reverse transcription–PCR (RT-PCR) specific for members of the genus *Flavivirus* (*2*). The RT-PCR product was sequenced, and according to BLAST (www.ncbi.nlm.nih.gov/blast), the 184-bp sequence obtained shared 99% nt identity with dengue serotype 3 viruses in GenBank. The plasma sample also had positive results for dengue virus nonstructural protein 1

(NS1) antigen test (Platelia NS1 Ag ELISA; Bio-Rad, Marnes-la-Coquette, France), anti-dengue immunoglobulin (Ig) M ELISA (Focus Technologies, Cypress, CA, USA), and in an in-house IgG immunofluorescence assay that used DENV-3–infected Vero E6 cells as antigens (titer 40). Other concomitant infections were ruled out by bacterial cultures and by laboratory tests for various viral, bacterial, and parasitic pathogens.

Virus isolation was conducted as described (*3*). Viral RNA was extracted from the supernatant of the infected Vero E6 cells, and the envelope gene was amplified in an RT-PCR. The amplified product was directly sequenced (details available from P.R. upon request). The obtained envelope gene sequence (GenBank accession no. HQ336219) of 1,479 bp was aligned with 26 other DENV-3 strains, including the most similar sequences identified in nucleotide BLAST search and a global set of sequences representing different genotypes (*4*), by using MUSCLE (www.ebi.ac.uk/Tools/muscle/index.html). A neighbor-joining phylogenetic tree was inferred by using p-distance, with 1,000 bootstrap replicates in MEGA version 4 (www.megasoftware.net).

The strain isolated from Yemen in 2010 (this study) shared highest nucleotide homologies with strains from Jeddah, Saudi Arabia (98%–99%), and Tanzania (98%) and was phylogenetically grouped within genotype III of DENV-3. The most closely related strains also included recent isolates from Côte d'Ivoire, People's Republic of China, Bhutan, and India (Figure).

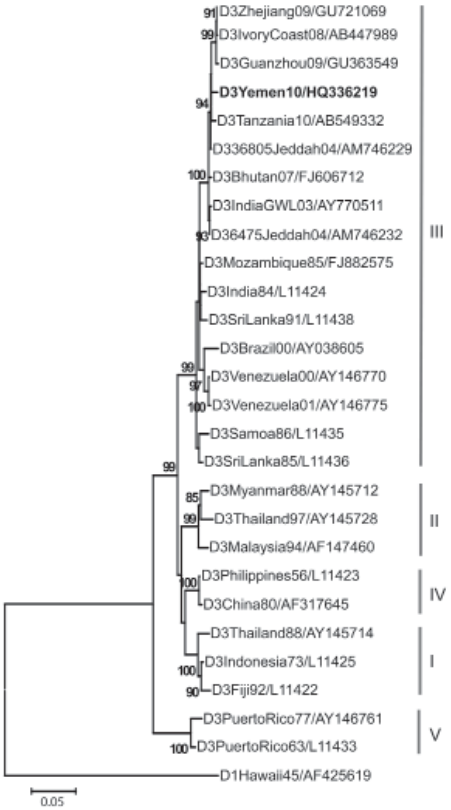

Figure. Neighbor-joining phylogenetic tree based on complete envelope gene sequences of dengue virus (DENV) serotype 3 virus, rooted with DENV-1. Bootstrap support values >80 are shown. **Boldface i**ndicates the 2010 isolate from Yemen. Scale bar represents nucleotide substitutions per site. Virus abbreviations are dengue virus type/origin/year/GenBank accession number.

Dengue has been documented in the Middle East, including Saudi Arabia (*5*) and Yemen (*6*). In May 2010, a dengue outbreak in Yemen was reported (*7*). The patient reported here had visited Yemen in June, and by August the outbreak had resulted in ≈100,000 infections and 200 deaths (*7*). To our knowledge, the DENV strains involved in this outbreak had not been previously characterized. The same genotype as the isolate described here, genotype III of DENV-3, was most recently isolated from Saudi Arabia in 2005 and has been associated with recent outbreaks in Sri Lanka, East Africa, and Latin America (*8*). Without further information, it remains unknown whether other serotypes or genotypes circulate concurrently in Yemen.

According to the Italian Public Health Institute (Istituto Superiore di Sanitá), the numbers of imported dengue cases in Italy are increasing (www.iss.it/binary/publ/cont/09_11web.pdf), but because only a few hospitals perform diagnostic tests, dengue is likely to be underdiagnosed in Italy. Viremic travelers can contribute to spread of DENV, and during the active mosquito season, travelers from dengue-endemic areas who have diagnosed or suspected dengue should be advised to avoid contact with mosquitoes.

Recently, indigenous transmission of dengue virus was shown to have occurred in Côte d'Azur in southern France (*9*) and in Croatia (*10*), thereby highlighting the risk in areas that have *Aedes albopictus* mosquitoes, which are competent DENV vectors. In these areas, including Italy, vector control and surveillance of DENV in mosquitoes should be conducted. We conclude that recognition and diagnosis of dengue in travelers should be emphasized and that characterization of DENV strains from travelers helps elucidate the molecular epidemiology of DENV in a global context.

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Strongyloidiasis in Man 75 Years after Initial Exposure

To the Editor: Strongyloidiasis, caused by the roundworm *Strongyloides stercoralis,* affects 100–200 million persons worldwide (*1*) and is endemic to Southeast Asia, sub-Saharan Africa, Latin America, and the southeastern United States (*2*). Endogenous autoinfection enables this nematode to develop into its host, which leads to the persistence of chronic infection several decades after a person has left a disease-endemic area (*3*). We encountered a case of prolonged strongyloidiasis with an infection going back >75 years.

An 83-year-old man who lived in Paris and had no medical history sought treatment for fatigue and weight loss. Results of a clinical examination were normal. Laboratory investigations showed mild hyperleukocytosis (12×10^9 cells/L) with hypereosinophilia (2.4×10^9 cells/L), which was observed for >3 months. Results of stool examinations were negative for parasites. Because of the patient's poor condition, cancer or hematologic malignancy were suspected, but results of various examinations, including a computed tomography scan of the body and bone marrow aspiration, were normal.

After several weeks, the patient disclosed that he had spent a few years in Vietnam >75 years ago. The only other travel abroad reported by the patient was a 10-day stay in a tourist hotel in the Canary Islands 15 years before this illness. At this point in his assessment, results of serologic testing were positive for *Strongyloides* spp., and a new stool examination showed *Strongyloides* larvae. Serologic test results were negative for human T-cell lymphotropic virus type 1. The patient received 2 doses of 12 mg of ivermectin within 15 days and fully recovered. Hypereosinophilia and *Strongyloides* larvae in feces disappeared.

S. stercoralis roundworms are ubiquitous intestinal parasites, endemic to tropical and subtropical regions. The larvae can develop into filariform larvae, which can penetrate the human skin and migrate through circulation to the lungs before settling in the intestine. In the human host, adult parasites may be generated by parthenogenesis in the mucosa of the small intestine. The resultant larvae can also penetrate the skin or the intestinal mucosa to establish a cycle of repeated endogenous reinfection. The parasite may then cause a long-lived autoinfection in the host, leading to chronic infection that can last for several decades (*3*,*4*).

Immunocompetent persons are usually asymptomatic and periodically exhibit eosinophilia. In

immunocompromised patients, the endogenous autoinfection cycle may result in the overproduction and dissemination of larvae into intestinal and extraintestinal tissues, including the central nervous system, leading to the hyperinfection syndrome which can be lethal (*5*). Most cases (96%) occur in immigrants, but some have been described in patients with a history of travel, sometimes many years previously. *S. stercoralis* infections have been reported up to 65 years after initial exposure in veterans who served in Asia during World War II (*4*,*6*).

Although our patient exhibited poor general condition, he likely did not experience hyperinfection syndrome because he was not immunosuppressed, and he completely recovered after receiving standard ivermectin treatment. That the patient was originally infected in the Canary Islands seems improbable, although a low level of transmission exists in rural and disadvantaged areas in continental Spain, Portugal, and Italy (*7*). We did not find evidence of *Strongyloides* spp. transmission in the Canary Islands. In particular, the patient stayed in a high-status tourist hotel for a short period, and he never walked in bare feet. He was probably infected when he lived in Vietnam.

This case highlights the importance of systematically considering chronic strongyloidiasis when seeking a diagnosis for persistent hypereosinophilia, even in patients with no underlying disease, and the value of systematically obtaining any history of travel in disease-endemic areas even if it occurred many years previously. The endogenous autoinfection cycle can possibly persist for a lifetime. In addition, systematic examination of stool samples should be carried out, and ivermectin should be given when an immunosuppressive drug is required in a patient who has a history of travel to, or residence in, an area to which strongyloidiasis is endemic.

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Anaplasma phagocytophilum Infection in Ticks, China–Russia Border

To the Editor: *Anaplasma phagocytophilum*, an emerging human pathogen of public health importance, is transmitted to humans most commonly by tick bites (*1*). The agent has been detected in various species of *Ixodes* ticks around the world (*2*) and in *Dermacentor silvarum* ticks in northeastern People's Republic of China (*3*), where 3 *A. phagocytophilum* strains were isolated from wild and domestic animals (*4*). In the Asiatic region of Russia adjacent to China, *A. phagocytophilum* was identified in *Ixodes persulcatus* ticks, and *A. bovis* in *Haemaphysalis concinna* ticks (*5*). Human granulocytic anaplasmosis was reported in the southern area of the Russian Far East that borders China (*6*). The objectives of this study were to investigate the prevalence of *A. phagocytophilum* in ticks collected from the China–Russia border and to characterize the agent by molecular biology techniques.

During May–June 2009, host-seeking ticks were collected by flagging vegetation of grassland or woodland along the China–Russia border. Attached ticks were collected from sheep and goats in Hunchun, and from dogs in Suifenhe (Table). All ticks were identified by morphologic features to the species level and the developmental stage by 2 entomologists (Y. Sun and R.-M. Xu). DNA was extracted from tick samples by using Tissue DNA Extract kit (Tiangen Biotechnique Inc., Beijing, China), following the instructions of the manufacturer. Nested PCR was performed to amplify partial citrate synthase gene (*glt*A) of *A. phagocytophilum* as previously described (*7*). To avoid possible contamination, DNA extraction, the

reagent setup, amplification, and agarose gel electrophoresis were performed in separate rooms, and negative control samples (distilled water) were included in each amplification.

A. phagocytophilum was detected in 83 of 2,429 adult ticks, with an overall prevalence of 3.42% (Table). The infection rates in the 14 survey sites ranged from 0 to 5.96%, and were significantly different (χ^2 = 24.43, df = 13; p = 0.027). Except for *H. japonica*, ticks from 4 species, including *H. concinna*, *H. longicornis*, *I. persulcatus*, and *D. silvarum*, were found to be naturally infected. The difference in infection rates among tick species was statistically significant (χ^2 = 13.03, df = 4; p = 0.011). Of 367 attached *H. longicornis* ticks obtained from domestic animals in Hunchun and Suifenhe, 12 (3.27%) were infected with *A. phagocytophilum* (Table). Nymphal ticks were only collected from vegetation in Hunchun, and 30 pools (10 in each pool) of 1,190 *H. concinna* nymphs were positive with an estimated minimum prevalence of 2.52%.

PCR products were purified by TIANgel Mini Purification Kit (Tiangen Biotechnique Inc.) and sequenced. The sequences obtained were compared with previously published sequences deposited in GenBank by using BLAST (http://blast.ncbi.nim.nih.gov/ Blast.cgi). The sequences of partial *glt*A from positive samples had 97.1%–100.0% identity in nucleotide sequences and 95.9%–100.0% identity in deduced amino acid sequences, 97.0%–99.4% and 96.5%–99.1% identity to Khabarovsk-01 strain from far eastern Russia (GenBank accession no. AY339602), and 96.1%–97.4% and 93.8%–98.3% identity to other corresponding sequences deposited in GenBank. Eighteen representative variant sequences obtained in this study were included in phylogenetic analysis based on 348-bp nucleotides of *glt*A by using neighbor-joining methods in MEGA 3.0 software (*8*), which found that the *A. phagocytophilum* identified in this study can be placed in a separate clade, together with Russian Khabarovsk-01 strain, which is distinct from previously reported strains from the United States and Europe (online Appendix Figure, www.cdc.gov/EID/content/17/5/932-appF.htm).

Table. *Anaplasma phgacytophilum* infection in adult ticks from the China–Russia border, 2009*

			Tick species, no. positive/no. tested (%)					
Survey site	Location	Origin	*Haemaphysalis concinna*	*H. longicornis*	*H. japonica*	*Ixodes persulcatus*	*Dermacentor silvarum*	Total
Mohe	52°28.34′N, 123°28.56′E	Woodland	–	–	–	2/49	0/6	2/55 (3.64)
Heihe	52°28.34′N, 123°28.56′E	Grassland	–	–	–	–	0/76	0/76
Jiayin	50°14.19′N, 127°26.39′E	Woodland	2/36	–	–	0/13	0/2	2/51 (3.92)
Xunke	49°34.26′N, 128°28.29′E	Woodland	0/98	–	–	0/3	0/70	0/171
Luobei	48°52.41′N, 130°03.56′E	Woodland	0/50	–	0/19	0/3	2/103	2/175 (1.14)
Tongjiang	47°34.54′N, 130°15.2′E	Woodland	0/4	–	0/20	0/3	0/12	0/39
Huyuan	47°42.42′N, 131°28.37′E	Woodland	1/23	–	–	0/5	–	1/28 (3.57)
Raohe	48°18.05′N, 134°.20.26′E	Woodland	0/30	–	–	4/90	0/4	4/124 (3.22)
HuLin	46°49.48′N, 133°59.11′E	Woodland	0/36	–	–	0/18	4/89	4/143 (2.80)
Mishan	45°50.8′N, 133°09.04′E	Woodland	3/55	–	–	1/29	0/6	4/90 (4.44)
Mudanjiang	45°16.25′N, 131°58′E	Grassland	4/40	–	–	8/120	0/40	12/200 (6.0)
Dongling	44°31.23′N, 130°34.25′E	Woodland	7/261	–	–	1/48	0/44	8/353 (2.27)
Suifenhe	43°53.23′N, 130°46.46′E	Woodland	3/28	–	–	2/53	0/4	5/85 (5.89)
		Dogs	0/52	2/11	–	2/9	–	4/72 (5.56)
Hunchun	44°21.39′N, 130°44.3.18′E	Woodland	23/243	1/148	–	0/9	0/3	24/403 (5.96)
		Goats, sheep	0/4	10/356	–	1/2	0/2	11/364 (3.02)
Total			43/960 (4.48)	13/515 (2.52)	0/39	21/454 (4.63)	6/461 (1.30)	83/2,429 (3.42)

*–, none identified.

A. phagocytophilum infection has been reported in *I. persulcatus* and engorged *D. silvarum* ticks in northeastern China (*3*). In this study, we also found *Haemaphysalis* spp. ticks, including *H. longicornis* and *H. concinna* ticks, to be infected by the agent. This finding indicates that various tick species may be involved in the maintenance and transmission of *A. phagocytophilum*. Both *H. longicornis* and *H. concinna* ticks usually have 3 hosts in their life cycle and can infest a variety of wild and domestic animals such as rodents, deer, scaly anteaters, sheep, goats, and dogs. *Haemaphysalis* ticks are distributed in a broad range of China and sometimes feed on humans. Their competency as a vector for *A. phagocytophilum* and the importance of this agent in public health as well as in veterinary medicine has yet to be investigated, particularly in the areas where they are predominant (*9*). The *gltA* sequence analyses indicated that the agents detected in this study were similar to the strains isolated from rodents and sheep in northeastern China (*4*) and to *A. phagocytophilum* strains from the Russian Far East adjacent to our survey sites. However, the strains from China are genetically distant from *A. phagocytophilum* strains in the United States and Europe. The genetic diversity of *A. phagocytophilum* in various geographic locations deserves further study.

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Japanese Encephalitis, Tibet, China

To the Editor: Tibet is located in the Qinghai-Tibet Plateau of western People's Republic of China and has been internationally recognized as a Japanese encephalitis (JE)–nonendemic area because the average altitude is thought to be too high to facilitate the cycle of Japanese encephalitis virus (JEV) between mosquitoes and vertebrates (*1*,*2*). In addition, JE is a reportable infectious disease in China, and no clinically confirmed case has been reported in Tibet since establishment of a national case reporting system in 1951 (*3*,*4*). Neither the mosquito vector of JEV nor JEV isolates have been described in Tibet. In this study, JEV was isolated from *Culex tritaeniorhynchus* mosquitoes, the main vectors of JEV, collected in Tibet. Serologic assays detected anti-JEV antibodies in a large number of human and porcine serum samples collected in this region. These data demonstrate that JEV is currently circulating in Tibet.

During August 5–15, 2009, mosquitoes were collected in Mainling

County (altitude 2,900 m) and Medog County (altitude 1,000 m) in the Nyingchi area of Tibet. A total of 4,089 mosquitoes representing 7 species (*Cx. tritaeniorhynchus, Cx. pipiens pallens, Cx. bitaeniorhynchus, Armigeres obturbans, Anopheles maculatus maculates, An. peditaeniatu*, and *Aedes albopictus*) from 4 genera were collected in this study. The dominant mosquito species detected in Medog County was *Cx. tritaeniorhynchus* (2,442 [71.1%] of 3,436 mosquitoes collected there) (Table); no previous reports have described this species in Tibet. A total of 653 mosquitoes were collected in Mainling County, of which 489 (74.9%) were *Armigeres obturban*. No *Cx. tritaeniorhynchus* mosquitoes were collected in Mainling County.

Mosquitoes were homogenized in 97 pools by using TissueLyser (QIAGEN, Hilden, Germany) and screened with reverse transcription–PCR (RT-PCR) by using seminested primers designed to detect the JEV *PreM* gene (*5*). One *Cx. tritaeniorhynchus* pool, XZ0938, collected in Medog County was positive by PCR. Isolation of virus was conducted from PCR-positive sample by injecting mosquito homogenate supernatants into monolayers of BHK-21 and C6/36 cells. The supernatant of pool XZ0938 caused cytopathic effects in BHK-21 and C6/36 cells in successive cell passages. The complete genome of 10,965 nt was sequenced (GenBank accession no. HQ652538) as described (*6*), which included a 96-nt 5′ nontranslated region and a 570-nt 3′ nontranslated region. The single open reading frame coded for a polyprotein of 3,432 aa. Compared with the complete genome sequences of 62 known JEV isolates, the nucleotide sequence identity varied from 83.6% to 97.8% and amino acid sequence identity from 94.9% to 99.7%. Phylogenetic trees derived from nucleotide sequences of the complete genome of JEV strains indicated that XZ0938 was a member of genotype I JEV. A more detailed analysis indicated that the Tibet JEV is most closely related to JEV isolates KV1899 (1999, Korea, AY316157), and JEV/sw/Mie/41/2002 (2002, Japan, AB241119) (data not shown).

To determine whether local residents were infected by JEV, 248 human serum samples were collected in Mainling and Medog Counties from healthy persons. Neutralizing antibody against JEV was tested by 90% plaque-reduction neutralization tests by using standard methods (*7*). Serum samples were tested with serial 2-fold dilutions from 1 to 5. Diluted serum was mixed with equal volumes of culture medium containing JEV P3 strain. The samples were considered positive when the neutralizing antibody titers ≥10. Sixty-eight positive samples were determined by 90% plaque-reduction neutralization tests, which constituted 68 (27.4%) of all 248 serum samples. Twenty-two (22.0%) of 100 and 46 (31.1%) of 148 serum samples in Mainling and Medog Counties, respectively, were positive (Table). Currently, the local population is not vaccinated against JEV (*8*) because Tibet is considered a JE-nonendemic area (*1–4*). The observation that 68 (27.4%) of 248 serum samples from healthy humans contained neutralizing antibody against JEV at titers ≥10 suggests that this population is subject to substantial levels of subclinical JEV infection.

To determine the present situation of JEV infection in local pigs, we analyzed 66 serum samples collected from piglets 1–6 months of age in Mainling and Medog Counties; immunoglobulin M antibodies against JEV were detected by capture ELISA as described (*9*). That 22 (33.3%) of 66 piglet serum samples were positive for immunoglobulin M against JEV suggested that local pigs have been newly infected by JEV in 2009 and have participated in the cycles of JEV in the local area (Table).

JE is a global public health issue that has spread to >20 countries in Asia (*6*,*10*). In this study, we present evidence that JEV has extended its geographic range to Tibet, a region that previously was believed to be free of JE because of its elevation. Factors such as global warming, increased pig farming, and increased tourism and transportation may have contributed to the emergence of JE in Tibet. Conditions in Tibet, including the presence of the primary vector (*Cx. tritaeniorhynchus* mosquitoes), abundant amplification hosts (pig), and a naive population that has not been vaccinated against JEV, present the possibility for JE outbreaks. Increased surveillance for JE in this region is needed.

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Table. Results from testing of mosquitoes, humans, and pigs for JEV, Nyingchi area, Tibet, People's Republic of China, 2009*

	Mosquitoes		Humans		Pigs	
Collection sites	No. collected	*Culex tritaeniorhynchus*, no. (%)	No. samples	Neutralizing antibody titers against JEV ≥10, no. (%)	No. samples	JEV IgM antibody positive, no. (%)
Mainling County	653	0	100	22 (22.0)	30	17 (56.7)
Medog County	3,436	2,442 (71.1)†	148	46 (31.1)	36	5 (13.9)
Total	4,089	2,442 (59.7)	248	68 (27.4)	66	22 (33.3)

*JEV, Japanese encephalitis virus; Ig, immunoglobulin.
†Pool was positive for JEV by PCR.

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Babesia sp. EU1 Infection in a Forest Reindeer, the Netherlands

To the Editor: Fatal piroplasmosis in domestic reindeer (*Rangifer* spp.) was first reported by Kertzelli in 1909; he named the piroplasm *Piroplasma tarandi rhangferis*. Similar piroplasms also were observed in blood smears of reindeer that had a condition known as spleen disease, which occurred in the second part of summer in the Arctic tundra and was characterized by clinical signs such as splenomegaly, icterus, pale mucous membranes, and death (*1*). Hemoglobinuria, a characteristic sign of babesiosis, is not mentioned in these early 20th century reports. However, these signs were observed in a *Babesia divergens*–infected reindeer herd in Scotland (*2*).

The only other reported cases of severe babesiosis in reindeer and caribou (*Rangifer tarandus caribou*) were caused by *B. odocoilei*, a predominantly nonpathogenic parasite of white-tailed deer (*Odocoileus virginianus*) that can cause fatal infection in reindeer (*3*,*4*). *Babesia* sp. EU1 is a recently recognized zoonotic *Babesia* species that has been associated with human babesiosis in Europe and is phylogenetically related to the *B. odocoilei* parasite (*5*). We report on a juvenile reindeer with babesiosis caused by *Babesia* sp. EU1.

A 5-week-old, captive-bred, female forest reindeer from an otherwise healthy herd of 9 animals in a zoo in the Netherlands was euthanized after showing clinical signs of lethargy, jaundice, and hemorrhagic diarrhea for >8 hours that did not improve after treatment with butylscopolamine (Buscopan; Boehringer Ingelheim, Alkmaar, the Netherlands) and enrofloxacin (Baytril; Bayer, Leverkusen, Germany). At necropsy, jaundice was evident in the sclera, aorta, and

leptomeninges. On the basis of the degree of fat storage and muscle development, the body condition was fair. The lungs were hyperemic and edematous, and the trachea contained foam. The liver was enlarged and pale; the spleen was enlarged. The kidneys were dark brown. Hemoglobinuria was noted in the urinary bladder.

Tissue samples from various organs were in fixed in 4% phosphate-buffered formalin, embedded in paraffin, cut into 4-µm sections, and stained with hematoxylin and eosin. No microscopic lesions were found in the skin, thymus, thyroid gland, tonsils, salivary glands, tongue, gastrointestinal tract, or heart. Numerous hemosiderin-laden macrophages were found in the spleen and liver sinusoids. Pigmentary nephrosis with moderate tubular degeneration and focal interstitial petechial hemorrhages were seen in the kidneys. Erythrophagocytosis was evident in the mesenteric lymph nodes, liver, and spleen.

Cytologic analysis was performed on samples from the brain, liver, spleen, lungs, and large intestinal contents, which were stained with Hemacolor (Merck, Darmstadt, Germany). Large (2–3 µm), intraerythrocytic protozoal inclusions consistent with *Babesia* spp. (Figure) were identified in the liver, spleen, lung, and brain.

DNA was extracted from 200 µL blood and ≈25 mg of the tissues collected during the necropsy: bone marrow, brain, heart, kidney, liver, lung, lymph node, small intestinal wall, and spleen. All extractions were performed by using the Nucleospin Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The detection of PCR products by reverse line blot hybridization was performed as described (*6*). All blood and tissue samples from each organ tested were positive only for *Babesia* sp. EU1.

To confirm these results, primers 18SAN and 18SBN were used to amplify a 1,705-bp fragment of the 18S rRNA gene (*7*), the fragment was subsequently purified, cloned into the pGEM-T Easy Vector (Promega, Leiden, the Netherlands), and sequenced (Baseclear, Leiden, the Netherlands). The resulting sequence (GenBank accession no. GQ888709) was 100% identical to that of *Babesia* sp. EU1 isolated from human babesiosis patients from Italy and Austria (GenBank accession no. AY046575). In an attempt to identify subclinical carriers of this piroplasm, blood samples from the reindeer's mother and another nonrelated calf from the herd were collected and tested by reverse line blot, but test results for both animals were negative.

These findings make transplacental transmission as a route of infection less likely and favor the bite of an infected tick as the cause of disease. *Babesia* sp. EU1 is transmitted by *Ixodes ricinus* ticks (*8*), which are widespread in cool humid areas of Europe. Of *I. ricinus* ticks from the Netherlands, ≈1% are infected with *Babesia* sp. EU1 (*6*). The only confirmed reservoir host of *Babesia* sp. EU1 is roe deer (*Capreolus capreolus*) (*9*). The infected forest reindeer resided in a zoo in an area without direct contact with roe deer, although roe deer are abundant in the forests surrounding the zoo.

Because *Babesia* sp. EU1 can be transmitted both transovarially and transstadially (*10*), the infection source may have been the offspring of a tick infected in a previous generation or an immature tick that fed on infected roe deer outside the zoo or an as yet unidentified reservoir host and was carried into the reindeer's compound by hosts, such as birds or small rodents. *Babesia* sp. EU1 is the third *Babesia* spp. to be recognized as the cause of fatal babesiosis in reindeer, together with *B. divergens* and *B. odocoilei*.

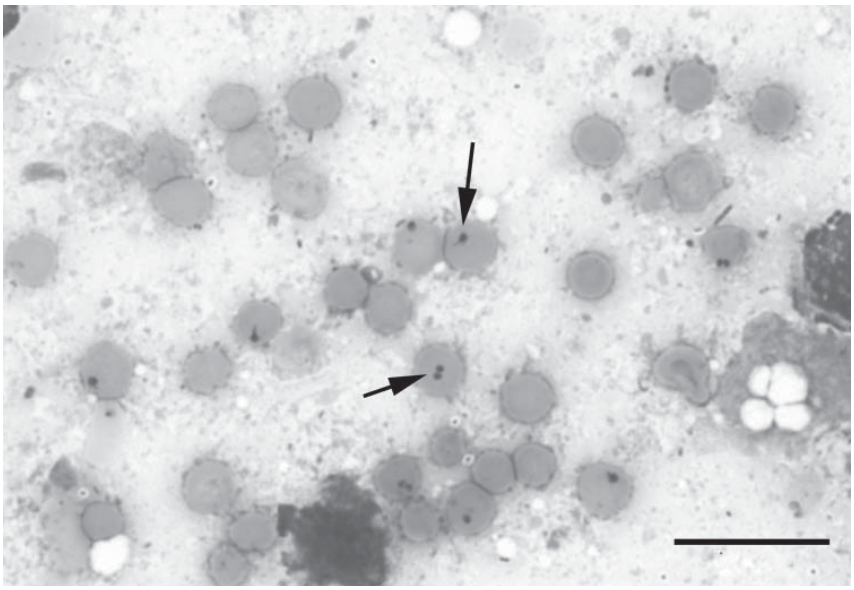

Figure. Lung of a forest reindeer infected with *Babesia* sp. EU1. Arrows indicate erythrocytes with protozoal inclusions. Scale bar = 20 µm.

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Dengue Virus Serotype 4, Roraima State, Brazil

To the Editor: In July 2010, dengue virus serotype 4 (DENV-4) reemerged in Boa Vista, the capital of Roraima State, in northern Brazil (Figure, panel A), after an absence of 28 years (*1*). Cases were identified during late June in the municipalities of Boa Vista and Cantá. For all patients, the clinical course of disease was classic, and all recovered uneventfully. The most commonly reported signs and symptoms were fever, headache, chills, muscle and joint pains, rash, nausea and vomiting,

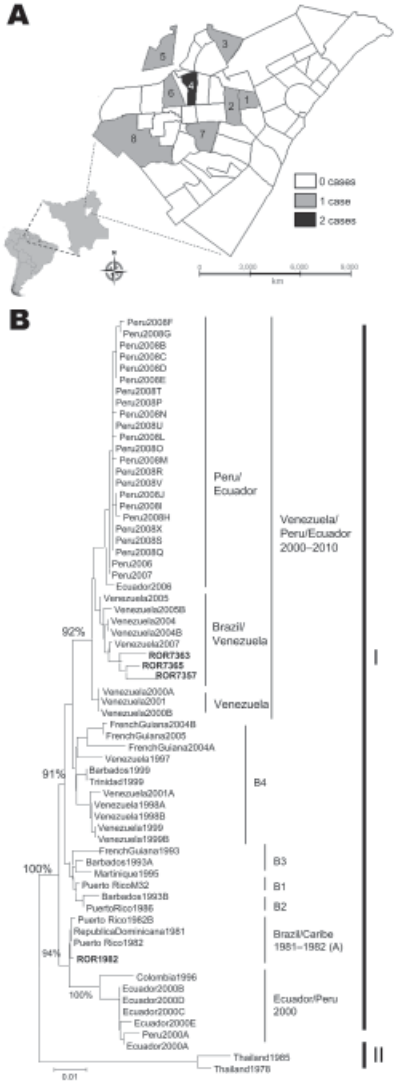

Figure. A) Boa Vista, Roraima State, Brazil, showing the districts where dengue virus type 4 (DENV-4) was isolated. 1, Liberdade; 2, Buritis; 3, Cauamé; 4, Santa Tereza; 5, Cidade Satélite; 6, Dr. Silvio Leite; 7, Joquel Clube; 8, Sen. Hélio Campos. B) Phylogenetic analysis of the DENV-4 envelope gene sequences (in **boldface**) constructed by using the neighbor-joining method, showing the cluster formed by Boa Vista and Venenzuela DENV-4 genotype I strains. Bootstrap values were set for 1,000 replicates and are placed over each main node of the tree. ROR1982 represents the DENV-4 isolated in Roraima State during the 1982 epidemic. A to B1–B4 represent genotype I subclades. Thailand 1978 and Thailand 1985 strains represent the Asian genotype II. Scale bar corresponds to 1% nucleotide sequence divergence.

and retro-ocular pain. Patient ages were 11–51 years (median 31 years); 5 patients were male.

Because of the clinical picture, dengue was suspected, and serum samples were collected and sent to Instituto Evandro Chagas for virus isolation. Of 10 DENV strains recovered, 9 were isolated into C6/36 cell samples as described elsewhere (*2*). Cytopathic effects were observed on days 5–7 postinfection, and virus isolation was confirmed by use of indirect immunofluorescent assay (*3*). Molecular approaches led to diagnosis of 10 cases. Viral RNA was recovered from infected cells in the supernatant by using a QIAquick viral RNA extraction kit (QIAGEN, Valencia, CA, USA); genome amplification was performed by using a 1-step reverse transcription–PCR (RT-PCR) (*4*) and 2 set of oligonucleotides designed to amplify the entire N gene in overlapping PCR products. PCR amplification was performed on 5 DENV strains, and the envelope gene (1,425 bp) was completely sequenced by the dideoxinucleotide terminator method for 3 strains (*5*) by using the same set of oligonucleotides as for the RT-PCR amplification.

Phylogenetic analysis was performed by using the neighbor-joining method (*6*) and homologous sequences of different DENV-4 strains isolated in Central and South America (*7*,*8*). The Asian genotype II strains (Thailand 1978-U18441 and Thailand 1985-AY780644) were used as outgroups to give confidence to phylogenetic groupings. Phylogenetically, the DENV-4 strains grouped in genotype I and clustered with Venezuelan strains isolated from 2004 through 2007 (Brazil/Venezuela group) and were distantly related to strains isolated in Venezuela from 1998 through 2000. This result indicates that the current DENV-4 strains isolated in Roraima State were reintroduced to Brazil through Venezuela, where DENV-4 has circulated since the 1980s (*1*). This result also excludes the possibility that Asian genotypes previously circulated in Brazil. The DENV-4 strains isolated from patients in Roraima State in 2010 were genetically distinct from DENV-4 strains isolated in the 1980s (Figure, panel B).

During the 2010 outbreak, cocirculation of DENV-1 and DENV-2 in addition to DENV-4 was demonstrated by virus isolation (68 strains) and RT-PCR amplification (genome detection in 39 strains). The municipality of Boa Vista, which has ≈266,901 inhabitants, reported 5,243 dengue cases (3,936 dengue fever, 259 severe dengue or dengue hemorrhagic fever) in 2010 (epidemiologic week 37), many of them diagnosed by serologic testing (*9*). These numbers represent an increase of 154% over the 2,066 cases reported in 2009 (*10*).

DENV-4 had been introduced to Brazil through Boa Vista before the reemergence reported here; in 1982, DENV-1 and DENV-4 were described in Brazil, and a serologic survey estimated 11,000 dengue infections (*1*). After that outbreak, DENV-4 was eradicated and not again detected until the episode reported here. To monitor the circulation of DENV-4 in Roraima and other Brazilian states through the present date (epidemiologic week 48), strong serologic and virologic surveillance have been conducted; cases of this serotype have not been recognized outside Roraima State. As a preventive measure in Boa Vista, a total of 10,358 dwellings were visited and 18,305 larval *Aedes aegypti* mosquito foci were eliminated by spraying of mosquito adulticide. Nonetheless, as summer approaches in Brazil, a heavy rainy season is expected, and DENV-4 is a candidate to become a serious threat in the country. Therefore, the Ministry of Health has prepared a plan to investigate DENV-4 circulation; it calls for early detection of disease and adoption of control measures to avoid or minimize spread of this dengue serotype throughout the country.

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Novel Phlebovirus in Febrile Child, Greece

To the Editor: Phleboviruses (family *Bunyaviridae*, genus *Phlebovirus*) are arthropod-borne, single-stranded, RNA viruses. Their genome consists of 3 segments—small, medium, and large—which encode the nucleoprotein and nonstructural proteins, the envelope glycoproteins, and the viral polymerase, respectively (*1*). The genus *Phlebovirus* consists at least 60 antigenically distinct serotypes, including the sandfly fever viruses transmitted to humans by phlebotomine sandflies. In the Mediterranean region, 3 phleboviruses are known to circulate: Toscana virus, sandfly fever Naples virus, and sandfly fever Sicilian virus. Sandfly fever Naples virus and sandfly fever Sicilian virus cause a transient febrile illness, whereas Toscana virus is sometimes neurovirulent, leading to aseptic meningitis and meningoencephalitis (*2*,*3*).

Phleboviruses have been detected in Greece in clinically ill persons and in sandflies; seroprevalance in humans is high, especially in the Ionian islands (*3*–*7*). In addition, in 2002, a Sicilian-like virus (Cyprus virus) was responsible for a major outbreak of febrile illness among Greek Army forces in Cyprus (*8*). We report genetic detection and sequencing of an Adria virus from a boy who was hospitalized because of simple febrile seizure; the identical sequence was initially detected in sandflies collected in a coastal area in Albania.

On September 23, 2009, a 2.5-year-old boy was admitted to the Hippokration Hospital of Thessaloniki, Greece, after a single episode of simple febrile seizure. The patient was febrile (38.2°C) and had vomited 1 time while in nursery school. He had sudden onset of eye gaze, perioral cyanosis, masseter muscle spasm, generalized tonic convulsions of the body and extremities, and involuntary loss of urine. The episode lasted ≈3 minutes, after which the child became irritable and sleepy.

At the time of hospital admission (30 minutes later), he was afebrile and conscious. The boy's history contained no previous neurologic or developmental disabilities and no family history of epilepsy or febrile seizures. Clinical examination, which included a thorough general and detailed neurologic evaluation, revealed no abnormalities except mild rhinitis. Laboratory tests showed leukocytosis (22,600 cells/μL) with 85.7% neutrophils. Blood levels of electrolytes, urea nitrogen, creatinine, glucose, albumin, bilirubin, alkaline phosphatase, and aminotransferases; prothrombin time; and urinalysis results were within reference limits. Electroencephalogram showed no brain abnormalities. Lumbar puncture and neuroimaging were not considered necessary.

After 2 days of hospitalization, the child recovered, was free of signs and symptoms, and was discharged from the hospital with a diagnosis of simple febrile seizure and mild upper respiratory infection. According to a report from his mother 1 year later, the child remains well without any recurrence of febrile or other type of seizures.

Viral RNA was extracted from the patient's blood sample taken at the time of hospital admission.

Table. Percentage nucleotide and amino acid sequence divergence among phleboviruses*

	Virus (GenBank accession no.)						
Virus	ADRV-GR	ADRV (HM043726)	SALV (GU143716)	ARBV (DQ862467)	SFSV (EF095551)	TOSV (FJ153280)	SFNV (EF095548)
ADRV-GR		0	21.6	29.6	82.0	84.8	96.4
ADRV	0		21.6	29.6	82.0	84.8	96.4
SALV	3.0	3.0		33.5	83.6	89.8	100
ARBV	17.7	17.7	17.5		75.5	73.6	96.4
SFSV	85.6	85.6	85.6	82.3		76.1	70.7
TOSV	78.3	78.3	76.3	73.1	75.7		47.4
SFNV	86.7	86.7	86.2	87.0	84.1	29.2	

*Values above the diagonal are nucleotide sequence divergence, and values below the diagonal are amino acid sequence divergence, estimated by neighbor-joining method. ADRV-GR, Adria virus from febrile child in Greece, 2009; ADRV, Adria virus; SALV, Salehabad virus; ARBV, Arbia virus; SFSV, sandfly fever Sicilian virus; TOSV, Toscana virus; SFNV, sandfly fever Naples virus.

Nested reverse transcription–PCR using degenerate primers was applied to amplify a 222-bp fragment of the large RNA segment of phleboviruses (*9*). The retrieved sequence was identical to sequences detected in sandflies collected in 2005 in the Adriatic coastal region of Albania; that strain was provisionally named Adria virus (*10*). Adria virus is distinct from other recognized members of the genus *Phlebovirus* and clusters with phleboviruses of the Salehabad serocomplex, such as Salehabad virus and Arbia virus, differing by 21.6% and 29.6% with Salehabad virus and Arbia virus at the nucleotide level and by 3% and 17.7% at the amino acid level, respectively (Table).

Detection of the Adria virus sequence in the patient's blood suggests that this virus is pathogenic to humans. As expected, serologic testing of the sample taken at the time of admission produced negative results for phleboviruses; a convalescent-phase blood sample was not available. Although the course of the disease in the child was mild, further studies will show the role of this strain in public health.

Because the duration of viremia in persons with phlebovirus infections is short, use of molecular methods for the laboratory diagnosis of phleboviral infections is limited; and even when a phleboviral infection is confirmed by serologic testing, the exact strain is difficult to determine. Physicians in Greece, as in other Mediterranean countries, should be aware of the circulation of phleboviruses and potential risk for phlebovirus-associated infections during the summer. Such infections, especially with neurologic signs, should be included in the differential diagnosis of summer febrile syndromes.

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Seroprevalence of Toscana Virus in Blood Donors, France, 2007

To the Editor: Toscana virus (TOSV) is an arthropod-borne RNA virus (family *Bunyaviridae* and genus *Phlebovirus*) transmitted by sandflies in Mediterranean countries. TOSV causes acute meningitis and meningoencephalitis in patients. In France, cases of TOSV infections involving resident populations and cases imported by tourists traveling in TOSV-endemic countries have been reported (*1*,*2*); the virus has also been isolated from local wild-caught sandflies (*1*). The fact that TOSV has been isolated from human blood on several occasions (*2*) suggests a potential risk exists for transmitting the virus through blood transfusion or organ transplantation. We investigated the presence of TOSV antibodies in a sample of the healthy population, blood donors from southeastern France.

We tested plasma collected from 729 blood donors in 7 French territorial divisions during the summer of 2007. Plasma donors were

analyzed according to their address of residence in each territorial division. Information related to these donors is reported in the Table.

Presence of immunoglobulin (Ig) G and IgM against TOSV was investigated by using a commercial enzyme immunoassay kit (EIA Enzywell Toscana virus IgG and IgM; DIESSE Diagnostica Senese S.p.A., Siena, Italy) developed by using the recombinant nucleocapsid (N) protein of TOSV. This serologic test was validated in a previous study that revealed high specificity and sensitivity (*3*).

Our results showed that 84 (11.5%) of 729 plasma samples were positive for IgG against TOSV N protein. Twenty-four (3.3%) plasma samples were positive for IgM, and 5 (0.7%) were positive for IgG and IgM (Table).

To confirm the ELISA results, IgG-positive samples were further subjected to Western blot (WB) analysis by using TOSV (isolate H/IMTSSA [*2*])–infected cell lysate (*4*). In 233 (32%) of samples, we detected a protein of molecular mass compatible with that of the N protein. A previously reported antibody-positive control was used to validate the WB assay (*5*). Our WB analysis showed a reduced sensitivity when compared with results of ELISA. After chemical/heat treatment of the protein samples, WB will only detect the linear epitopes on the N protein, while ELISA detects both linear and conformational epitopes. Furthermore, a less recent exposure of the blood donor population to the virus would have resulted in weaker N protein detection by WB as a consequence of a lower antibody titer. However, we cannot exclude some aspecific cross-reactivity as a consequence of well-conserved N protein sequence among the genus.

Finally, to detect TOSV RNA, we processed IgM-positive plasma samples by reverse transcription–PCR (*6*). The finding of IgM is an indication of a recent exposure to the virus and hence a possible presence in blood. Our PCR did not detect any viral RNA in the samples. Such negative results could indicate either cleared viremia or a low viral load, below the sensitivity limit of the test.

Serologic information obtained in our study confirms the circulation of TOSV in southeastern France. Factors such as commercial exchange and movement of humans, animals, and arthropods between France and Italy may explain the highest prevalence observed (18.8%) in the Alpes Maritimes territorial district, which borders Italy. Our results regarding this area appear of the same order of magnitude as those reported in the general Italian population (>20%) (*1*).

Geographic and climatic conditions (e.g., temperature, humidity), factors that affect vector distribution and abundance (*7*), could explain the lower prevalence found in the mountainous districts (collectively ≈400–2,000 meters in elevation). The lower temperatures in these districts may also affect the ability of vectors to efficiently transmit the virus in the field (*8*).

TOSV prevalence in Corsica, an island in the Mediterranean Sea, was unexpectedly high. In this region, ≈8.7% (10 donors of 115) of the population sampled showed an IgG- or IgM-positive response. In the other districts, the IgM seroprevalence did not exceed 4.4%. The vector that transmits TOSV is known to be present in this area (*7*), and TOSV infections have been reported on nearby Sardinia (*9*). The elevated IgM titer in the population in Corsica could indicate 1) recent virus contacts; 2) recent infections with a new TOSV strain circulating in Corsica; or 3) presence of related phleboviruses that are inducing cross-reactivity in the N protein–based IgM ELISA.

Our results demonstrate that 14.1% (IgG and IgM) of the healthy population (blood donors) in France living on the Mediterranean border

Table. Prevalence of antibodies against Toscana virus in blood donors, France, 2007*

Demographic characteristic	No. donors	% IgG-positive samples	% IgM-positive samples
Age, y			
<30	211	11.8	3.8
30–39	133	11.3	3
40–49	156	11.5	2.6
50–60	158	10.8	4.4
>60	71	12.7	1.4
Sex			
F	353	13.9	2.5
M	376	9.3	4
French territorial division†			
Alpes de Haute Provence	29	10.3	3.4
Hautes Alpes	64	6.25	1.6
Alpes Maritime	111	19	0
Bouches du Rhône	143	12.6	2.8
Corsica	115	8.7	8.7
Var	154	8.4	1.9
Vaucluse	113	13.3	4.4
Total	729	11.5	3.3

*Plasma samples were determined as positive by using an ELISA to detect immunoglobulin (Ig) G against Toscana virus (absorbance cutoff value [optical density at 450 nm (OD_{450})]>0.47) and IgM (absorbance cutoff value of OD_{450}>0.15). Mean age of seropositive blood donors were the following: women, 40 y (SD 13.73 y); men, 41.8 y (SD 13.71 y).
†French territorial division elevations: Alpes de Haute Provence, 280–3,412 m; Hautes Alpes, 430–4,101 m; Alpes Maritimes, 0–3,143 m; Bouches du Rhône, –2–1,042 m; Corsica, 0–2,706 m; Var, 0–1,714 m; Vaucluse, 12–1,909 m.

have been in contact with TOSV and show asymptomatic or mild, unidentified symptoms, as it is the case for many other arbovirus infections (*10*). Such findings raise concerns about the risks of virus transmission to virus-naive persons by blood transfusions and organ transplants.

Further investigation is needed to better assess how widespread TOSV is in populations. For example, a donor–recipient investigation might confirm virus transmission by blood transfusion, and studies related to the behavior of sandfly vectors, virus biology, and mammalian reservoir hosts could help define populations at higher risk for exposure.

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Quinine-Resistant Malaria in Traveler Returning from French Guiana, 2010

To the Editor: Resistance of *Plasmodium falciparum* to antimalarial drugs is one of the most worrying problems in tropical medicine. For *P. falciparum* malaria acquired in French Guiana, the combination of quinine and doxycycline is one of the first-line recommended treatments (*1*). Since 1996, only 2 treatment failures with quinine have been reported from that country (*2*). An elevated 50% inhibitory concentration (IC_{50}), classified as in vitro quinine resistance, was reported for 17% of 32 *P. falciparum* isolates obtained during 1983–1987 in French Guiana (*3*). Throughout 1994–2005, isolates were susceptible to quinine, with a mean IC_{50} <200 nmol/L (*4*).

We report quinine treatment failure in a 35-year-old man who was infected during a 3-month stay in Saül, a rural area of French Guiana. The patient did not use antivectorial or antimalarial prophylaxis. The patient sought treatment with fever 4 days after returning to France on June 22, 2010 (day 0), and a diagnosis of *P. falciparum* malaria was made on the basis of results of a rapid diagnostic test performed by a private medical laboratory. The man, who weighed 58 kg, was treated as an outpatient with 500 mg of quinine to be taken orally 3×/d for 7 days; he did not receive doxycycline. He was admitted to the Laveran Military Teaching Hospital in Marseille on July 15 (day 24 and first day of recrudescence) for uncomplicated malaria with a *P. falciparum* parasitemia level of 4%. He was given artemether, 80 mg/d, by intramuscular injection for 3 days. Blood samples taken on day 27 (third day of recrudescence) and day

52 (4 weeks of recrudescence) were negative for *P. falciparum*.

In vitro testing of drug susceptibility was performed by the standard 42-hour ^{3}H-hypoxanthine uptake inhibition method (*5*). We assessed susceptibility to 11 antimalarial drugs on the fresh isolate and after culture adaptation (Table). The laboratory-adapted strain 3D7, tested 3× on the same batch of plates, was used as reference. The strain isolated from the blood sample on day 24 (first day of the recrudescence) showed reduced susceptibility to quinine (1,019 nmol/L), chloroquine (427 nmol/L), and monodesethylamodiaquine (157 nmol/L). The isolate was susceptible to all other antimalarial drugs tested. We assessed gene polymorphisms of *pfcrt* (*P. falciparum* chloroquine resistance transporter), *pfmdr1* (*P. falciparum* multidrug resistance 1 protein), and *pfnhe-1* (*P. falciparum* Na^{+}/H^{+} exporter 1); the copy number of *pfmdr1* involved in quinoline resistance; and gene polymorphisms of *dhfr* (dihydrofolate reductase, involved in proguanil or pyrimethamine resistance), *dhps* (dihydropteroate synthetase, involved in sulfadoxine resistance), and *cytB* (cytochrome B, involved in atovaquone resistance) (*6*).

The *pfnhe* ms4760 microsatellite showed a profile 3, with 1 repeat of DNNND and 2 repeats of DDDNHNDNHNN. Studies of the *pfnhe-1* polymorphism of worldwide culture-adapted isolates showed that increased numbers of DNNND were associated with decreased quinine susceptibility (*7*). Association of 2 repeats of DNNND and a high quinine IC_{50} value was found in a case of clinical failure of quinine in a traveler returning from Senegal (*8*).

Reinfection was excluded because the patient had stayed in mainland France since his return. The patient reported that he took the quinine as instructed. We report here a clinical and parasitologic failure of quinine treatment associated with high IC_{50} but not linked with the ms4760 *pfnhe-1* profile involved with quinine in vitro reduced susceptibility. A hypothesis that may explain our data is that unlike previous studies (*7*), in which the less susceptible strains originated from Asia, this isolate came from South America. The profile associated with quinine resistance for chloroquine resistance and *pfcrt* genotypes could be different in the 3 malaria-endemic continents. There are no data on ms4760 *pfnhe-1* of *P. falciparum* isolates from South America. In another recent study (*9*), a multivariate analysis performed on 83 clinical isolates from Madagascar and 13 African countries did not confirm this association between quinine susceptibility and *pfnhe-1* microsatellite polymorphisms.

The *pfcrt* gene had a point mutation on codon 76 (76T) and *pfmdr1* on codons 184F, 1034C, 1042D, and 1246Y. These data are in agreement with those from previous studies that showed the mutation 76T in the *pfcrt* gene led to decreased susceptibility to chloroquine, amodiaquine, and quinine. The isolate had only 1 copy of *pfmdr1*. The data on mutations and copy number of *pfmdr1* are consistent with data in Brazil (*10*). Nevertheless, the lack of gene amplification and specific point mutations in *pfmdr1* were not associated with decreased in vitro susceptibility of quinine. *dhfr* and *dhps* genes had a 5-mutation haplotype, 51I C59 108N I164-S436 437G 540E 581G A613, which suggested in vitro resistance to proguanil, pyrimethamine, and sulfadoxine. This case confirms the need to always add doxycycline to quinine for treatment of *P. falciparum* malaria acquired in French Guiana as well as other parts of South America.

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Table. In vitro susceptibility to standard antimalarial drugs of a fresh isolate of *Plasmodium falciparum* and after culture adaptation in comparison with *P. falciparum* 3D7 clone tested with the same plate batches*

Antimalarial drug	Fresh isolate IC_{50}, nmol/L	Cultured isolate IC_{50}, nmol/L, mean ± SD†	3D7 clone IC_{50}, nmol/L, mean ± SD	IC_{50}, isolate:3D7	Resistance cutoff value, nmol/L
Quinine	1,019	1,087 ± 145	171 ± 26	6.0:6.4	>800
Chloroquine	427	492 ± 39	13.9 ± 2.2	30.7:35.4	>100
Monodesethylamodiaquine	157	157 ± 27	16.3 ± 3.5	9.6:9.6	>80
Mefloquine	16.4	14.9 ± 2.2	53.4 ± 5.2	0.31:0.28	>30
Lumefantrine	8.9	9.0 ± 3.0	46.0 ± 6.0	0.19:0.20	>150
Pyronaridine	45.2	37.8 ± 8.4	18.3 ± 1.6	2.5:2.1	ND
Piperaquine	80.4	99.5 ± 11.7	56.3 ± 5.4	1.4:1.8	ND
Dihydroartemisinin	2.18	2.46 ± 0.28	2.58 ± 0.13	0.84:0.95	>10.5
Artesunate	1.86	1.68 ± 0.28	2.27 ± 1.15	0.82:0.74	>10.5
Atovaquone	6.5	5.2 ± 0.8	4.2 ± 0.7	1.5:1.2	>490
Doxycycline	11,600	12,600 ± 1,700	12,600 ± 1,000	0.92:1.0	>35,000

*Isolate was obtained from a man who returned to France from French Guiana in June 2010. IC_{50}, 50% inhibitory concentration; ND, not determined.
†Mean IC_{50} and standard deviation of 3 different tests on the isolate after culture adaptation.

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Kyasanur Forest Disease Virus Alkhurma Subtype in Ticks, Najran Province, Saudi Arabia

To the Editor: The lineage of Kyasanur Forest disease virus (KFDV) found in the Kingdom of Saudi Arabia is commonly referred to as Alkhurma hemorrhagic fever virus (AHFV). This virus was first isolated from a specimen collected in 1994 from a butcher living in Makkah Province, who was hospitalized for a hemorrhagic fever from which he died (*1*). The virus was assigned to the genus *Flavivirus* on the basis of reactivity with genus-specific monoclonal antibodies and sequencing of a fragment of the nonstructural 5 (NS5) gene, which showed >89% identity with KFDV. Ten other cases were confirmed among patients who had leukopenia, thrombocytopenia, and elevated liver enzymes. Observations of patients in the original study or in a subsequent analysis (*2*) suggested that Alkhurma hemorrhagic fever (AHF) disease was associated with contact with blood from infected animals, bites from infected ticks, or the drinking of raw milk. However, the exact mode of transmission to humans has still not been fully elucidated. More recently, AHFV RNA was detected in a single pool of sand tampans (*Ornithodoros savignyi*, soft ticks), collected in western Saudi Arabia (*3*), which suggests a link with these ticks.

To analyze the virus association with arthropods further, we collected and identified ticks and mosquitoes in Najran Province, southern Saudi Arabia, during May and June 2009 from different sites close to where human AHF cases had been recently confirmed (*4*,*5*). Camel ticks (*Hyalomma dromedarii*) (130 adults) were collected while they fed on camels, and *O. savignyi* sand tampans (243 adults) were collected from the ground in camel resting places (except 1 collected while feeding on a camel). Mosquitoes were collected by using light traps (203 *Culex decens* females) or as larvae that were then raised in the laboratory (9 *Culiseta* sp. females). Ticks and mosquitoes were stored at room temperature and killed by overnight freezing the day before shipping to the Centers for Disease Control and Prevention (Atlanta, GA, USA). All arthropods were processed in the BioSafety Level 4 laboratory by injecting Vero E6 cells and by intracerebrally inoculating suckling mice with ground pools of either 5 ticks or 10 mosquitoes. All the tick material was used for the tested pools. Isolates of AHFV were obtained from 1 of 13 pools of *H. dromedarii* ticks and 1 of 6 pools of *O. savignyi* sand tampans, both from Al Mishaaliyia

district, and from 5 of 8 pools of *O. savignyi* sand tampans from the Al Balad Magan camel market. Virus identity was confirmed by sequencing a 390-nt fragment from the virus core protein C and preM genes. No virus was isolated from any mosquito suspensions.

Phylogenetic analysis of the 7 tick isolates and the available homologous sequences of AHFV are presented in the Figure. The tick AHFV sequences are closely related but distinct from previously reported AHFV sequences from human isolates or from the only sequence reported from ticks collected in 2004 in Jeddah Province. The observed sequences are clustered by site of collection but not by tick species.

In this report, we confirm that the sand tampan (*O. savignyi* tick) is a vector and reservoir of AHFV in Saudi Arabia. Of all arthropods, this tick is one of the most highly adapted to the desert. It can be found in the shade of trees, beside rock fences, on livestock, and in livestock yards, particularly camel yards (*6*). It can feed rapidly during the day or night on camels, goats, sheep, wild mammals, and humans. Sand tampans can survive for long periods without feeding, fulfilling perfectly the role of reservoir for AHFV. This tick has been reported in arid biotopes of northeastern, eastern, and southern Africa (*7*) and from Arabia to India and Sri Lanka, which suggests a potential wide distribution of AHFV or related viruses. In India, KFDV has been isolated from *Ornithodoros* spp. ticks collected in a bat-inhabited cave (*8*), and experimental transtadial and transovarial transmission of KFDV in *O. crossi* ticks has been reported (*9*).

The isolation of AHFV from the camel tick (*H. dromedarii*) also has public health implications. The capital city of Najran serves as a market for camels and other livestock from Saudi Arabia and Yemen. Adult camel ticks infest mainly camels, and infected ticks can feed on and infect animals just before sale or slaughter. AHF in persons working in the Najran market has been described (*5*). Unfortunately, no AHFV sequence is available from those cases.

The genetic diversity of the isolated viruses from ticks is quite low. Previous analysis of KFDV and AHFV suggested slow evolution with divergence ≈33 years ago (*10*). The data reported here clearly strengthen the position of AHFV in the tick-borne flavivirus complex, although the numbers and species of mosquitoes tested were limited. Expanded epidemiologic and molecular studies should provide insight into the distribution and evolution of the virus and identify at-risk regions within Saudi Arabia. Laboratory infection and transmission studies in colonized ticks should clarify the role of *O. savignyi* and *H. dromedarii* ticks in the ecology of AHFV. Currently, public health messages are being developed for the community at risk and local health care workers.

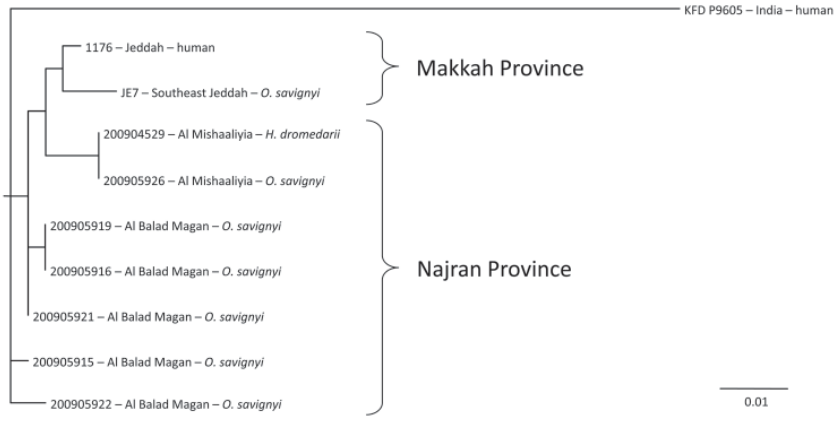

Figure. Phylogenetic analysis of Alkhurma viruses isolated from *Ornithodoros savignyi* and *Hyalomma dromedarii* ticks in Najran Province, Kingdom of Saudi Arabia. A 390-bp region of the core protein C and preM genes was amplified and sequenced for each of the isolates (HQ443410–6) by using primers ALK244S (5′-GTGTTGATGCGCATGATGGG-3′) and ALK665R (5′-TGCAGAAACAGTCCACATCA-3′). A maximum-likelihood analysis was conducted with available sequences in GenBank for ALK (NC_004355; *3*) by using Kyasanur Forest disease (AY323490) as the outgroup and the default settings in GARLI version 0.96b8 (www.phylo.org/pdf_docs/zwicklDissertation.pdf). Scale bar indicates substitutions per site.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

West Nile Virus Infection, Assam, India

To the Editor: West Nile virus (WNV) is a mosquito-borne flavivirus. Sporadic infections with this virus have been found in Africa, Europe, Asia, and the United States. In humans, most infections with WNV cause subclinical or a mild influenza-like illness; encephalitis occurs in some (*1*). In India, antibodies against WNV were first detected in humans in Bombay in 1952 (*2*). Virus activity has been reported in southern, central, and western India. WNV has been isolated in India from *Culex vishnui* mosquitoes in Andhra Pradesh and Tamil Nadu, from *Cx. quinquefasciatus* mosquitoes in Maharashtra, and from humans in Karnataka State (*3*).

Assam (26°–27°30′N, 89°58′–95°41′E) is the most populated state in northeastern India; it contains ≈50% of the 38.8 million inhabitants of northeastern India. Japanese encephalitis virus (JEV) has caused sporadic epidemics in Assam since 1976. Studies conducted during 2000–2002 in Assam showed that 187 (53.7%) of 348 persons with acute encephalitis syndrome were infected with JEV (*4*). JEV-negative persons also showed symptoms of neurotropic viral infection.

Suspecting the presence of some other closely related flavivirus in this region, we screened samples from persons with acute encephalitis syndrome for WNV in 2006. To our knowledge, no study has been conducted on the prevalence of WNV in this region. We report WNV activity in the state of Assam in northeastern India. Ethical approval for this study was obtained from the institutional ethical committee, Regional Medical Research Center, Dibrugarh, India.

A JEV vaccination campaign (SA14-14-2 vaccine) was started in Assam during May 2006. During its first phase, children 1–15 years of age in Dibrugarh and Sivasagar Districts were vaccinated. Mosquito surveillance in the study area and in an earlier study (*5*) identified *Cx. vishnui* mosquitoes.

During the study period, 103 serum samples and 88 cerebrospinal fluid samples were obtained from 167 patients with acute encephalitis syndrome admitted to the Assam Medical College and Hospital in Dibrugarh, which administers to the health needs of ≥7 districts of Upper Assam and neighboring states of Arunachal Pradesh and Nagaland. Among the 167 patients, 124 (74.2%) were children ≤15 years of age.

Among the 103 serum samples, 80 were positive for immunoglobulin (Ig) M against JEV (IgM monoclonal antibody–capture ELISA; National Institute of Virology, Pune, India) and 12 (11.6%) were positive for IgM against WNV (IgM antigen-capture ELISA; Panbio, Sinnamon Park, Queensland, Australia). These samples were from persons in 4 districts in Assam (Dibrugarh, Golaghat, Sivasagar, and Tinsukia) and negative for IgM against JEV (Table). Follow-up was conducted for 9 patients; 3 died, and 1 was lost to follow-up.

Virus-neutralizing antibody titers against JEV and WNV were estimated in pig kidney epithelial cells by using JEV (isolate 733913) and WNV (isolate 68856) and a cytopathic-effect assay in 96-well tissue culture plates (*6*). Mouse polyclonal antibodies against JEV and WNV and nonimmune serum samples were included in the assay. Of 9 paired serum samples, 6 showed neutralizing antibody for WNV, of which 4 showed a 4-fold increase in antibody titer. The remaining 3 paired samples showed cross-reactivity with WNV (titer ≤80) and JEV (titer ≤40).

All 12 WNV-infected patients had high fever and headache. Convulsions (6 patients), altered sensorium (7 patients), vomiting (5 patients), and

Table. Incidence of JEV and WNV infections among patients with acute encephalitis syndrome, Assam, India*

District	No. with acute encephalitis syndrome	No. positive/no. tested	
		JEV	WNV
Dhemaji	1	0/1	0/0
Dibrugarh	29	9/29	6/29
Golaghat	81	47/81	2/18
Jorhat	15	8/15	0/15
Lakhimpur	6	5/6	0/6
Sivasagar	30	9/30	2/30
Tinsukia	5	2/5	2/5
Total	167	80/167	12/103†

*JEV, Japanese encephalitis virus; WNV, West Nile virus.
†One person was not included because the address could not be verified.

neck rigidity (2 patients) were also observed. Signs and symptoms at the time of hospitalization and at follow-up for 6 months (at 3-month intervals) were similar for persons infected with JEV and those infected with WNV. Neurologic sequelae observed at ≤6 months follow-up were impaired memory (6 patients), irritable behavior (5 patients), impaired hearing (3 patients), incoherent speech and disorientation (1 patient), breathing difficulty (1 patient), impaired speech (1 patient), and quadriparesis (1 patient).

We identified WNV in regions of Assam to which JEV is endemic. The finding indicates that WNV might be the cause of a substantial number of acute encephalitis syndrome cases in this region. Fever and headache were the most common signs and symptoms, as reported (*7*). There were 3 deaths (all children) in 13 patients. Our results corroborate a similar observation in the Kolar District of Karnataka (*8*). In contrast, in western countries, the attack rate and case-fatality rate for WNV infection are higher among immunocompromised elderly patients (*9*). Our findings may be caused by strain variations and host susceptibility to the virus. Identification of circulating genotypes of WNV and its vectors and epidemiologic studies are needed to obtain additional information on WNV infection in this region and identify WNV as a cause of acute encephalitis syndrome.

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Rare Rotavirus Strains in Children with Severe Diarrhea, Malaysia

To the Editor: We report the identification of G3P[9] rotavirus in children with acute diarrhea in Malaysia. Globally, rotavirus infections are the most common cause of severe diarrhea in infants and young children admitted to hospital. It is estimated that 527,000 children <5 years of age die each year of rotavirus diarrhea (*1*). Strains with a G3P[9] genotype represent a rare group of viruses, initially reported in Japan in 1982. These viruses have been sporadically associated with diarrhea in infants in countries such as Thailand, Italy, United States, Japan, Malaysia, and China (*2–7*) and thus represent a rare but widely distributed group of viruses.

Four genotype G3P[9] strains were identified from a total of 134 rotavirus-positive samples analyzed during surveillance studies conducted among children <5 years of age who were admitted to the University of Malaya Medical Centre, Kuala Lumpur, with acute diarrhea during 2008. To understand the possible origin of these G3P[9] viruses, we determined the sequence of the genes encoding the 2 outer capsid proteins, viral protein (VP) 7 and VP4, and analyzed their phylogenetic relationship to other rotaviruses.

Rotavirus double-stranded RNA was extracted by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), and the genes encoding the VP4 and VP7 proteins were amplified by reverse transcription–PCR (RT-PCR). The VP7 gene segment (nt 51–932) was amplified by using primers VP7-F and VP7-R (*8*), and the VP8 subunit of the VP4 gene (nt 150–795) was amplified by using the primers VP4-F and VP4-R (*9*). The PCR products were purified by using the QIAquick Gel Extraction Kit (QIAGEN) and sequenced by using the ABI Prism BigDye Terminator cycle sequencing kit version 3.1 (Applied Biosystems, Carlsbad, CA, USA) with primers homologous to both ends and internal regions of each gene. Sequencing was performed on an Applied Biosystems 3730*xl* DNA Analyzer at the Australian Genome Research Facility. Sequences were analyzed by using the Sequencher program version 4.1 (Gene Codes Corp., Inc., Ann Arbor, MI, USA), and aligned by using ClustalW (www.ebi.ac.uk/clustalw). Phylogenetic analysis was conducted by using MEGA version 4.1 and neighbor-joining method with 1,000 bootstrap replicates (*10*).The 4 G3P[9] rotavirus strains all exhibited identical nucleotide and amino acid sequences for the regions of VP7 and VP8 subunit of VP4 analyzed.

The VP7 gene from the G3P[9] strains from Malaysia exhibited greatest identity to VP7 genes from animal G3 rotaviruses; identities were 98% and 97% to a raccoon dog rotavirus (RAC-DG5, Japan, 2004) and a feline rotavirus (Australia, 1984), respectively. Comparison with the prototype G3P[9] strain AU-1 exhibited 90% nt homology. Notably, the VP7 gene of the Malaysian G3P[9] strain also shared 90% nt homology with human G3 strains isolated in Malaysia in 2004 and 2007. Phylogenetic analysis of the VP7 nucleotide sequence (nt 93–877) revealed that the Malaysian G3P[9] strain (552157) clustered with animal G3 strains and human G3P[9] strains from various countries but was distinct from G3P[8] strains causing disease in children in Malaysia over the same period (Figure, panel A).

Similar to the VP7 gene, the VP8 subunit of VP4 of the G3P[9] strains from Malaysia exhibited greatest nucleotide homology (98%) with the rotavirus strain isolated from a raccoon dog (RAC-DG5). High nucleotide homology of 96%–98% was also observed when the P[9] strain from Malaysia was compared with other human P[9] rotavirus strains isolated in Japan, Thailand, and China. Phylogenetic analysis revealed 3 distinct clusters among the VP8 sequences obtained from the P[9] strains (Figure, panel B). Human and animal P[9] strains from Asia grouped together within a single cluster. The

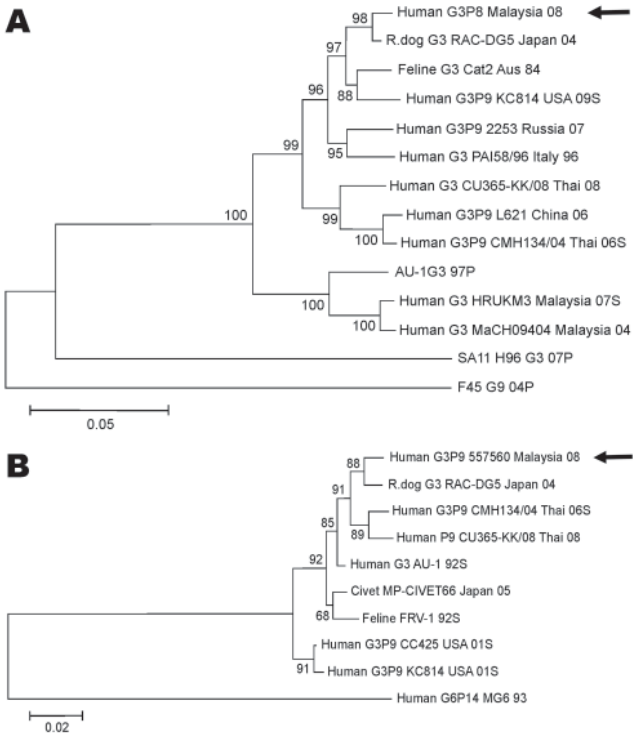

Figure. Phylogenetic relationship of nucleotide sequences of genes encoding the outer capsid proteins VP7 and VP4 from G3P[9] rotavirus strains. A) Evolutionary relationship of G3 VP7 nucleotide sequences. B) Evolutionary relationship of P[9] VP4 nucleotide sequences. The evolutionary relationship was inferred by using the neighbor-joining method. The percentages of the bootstrap test (2,000 replicates) are shown next to the branches. The evolutionary distances were computed by using the maximum-composite likelihood method and represent the number of base substitutions per site. Phylogenetic analysis was conducted by using MEGA version 4 (*10*). The labeling of the taxon corresponds to host name, followed by G-type and/or P-type, strain name, place of origin, and year of isolation. The letter S or P after the year of isolation indicates submission or published year of the sequence in the National Center for Biotechnology Information database. Arrows indicate G3P[9] isolate identified in this study. Scale bars indicate nucleotide substitutions per site.

feline strains from Italy and Australia grouped together, as did the P[9] strains from the United States.

Thus, both the VP7 and VP4 genes of G3P[9] strain identified in this study were most closely related to a racoon dog rotavirus strain (RACDG5), suggesting an animal origin of this rotavirus strain. These strains are likely an example of an animal strain causing limited disease in humans, rather than existence of a true strain, which has entered and adapted to the human environment. Recent whole-genome sequencing of 2 G3P[9] strains isolated from children in Italy showed they were composed of genes of human, bovine, and feline origin (*2*); whether the G3P[9] strains from Malaysia identified in this study are also human/animal reassortant strains requires further study.

Identification of G3P[9] strains in Malaysia continues to highlight the presence of these rare strains in Asian communities. The close similarity of the strains to a G3P[9] strain from a raccoon dog further highlights the transmission of rotavirus strains between animal and human sources. Whether this strain can establish itself in humans and cause disease is unknown, but the identification of rare strains illustrates that movement of rotaviruses between various hosts does occur from time to time.

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Avian Malaria Deaths in Parrots, Europe

To the Editor: Avian malaria is an insect-borne disease induced by a so far unknown number of protozoan blood parasites of the genera *Plasmodium* and *Haemoproteus* (hematozoa) (*1*,*2*). The unintentional introduction of *P. relictum* to the Hawaiian Islands, USA, has had fatal effects for the native bird fauna (*3*). In Europe, asymptomatic blood infections by hematozoa have been regularly observed, with an especially high prevalence in songbirds (*4*). However, numerous outbreaks of fatal protozoan infections have been reported over the past 40 years, mainly among psittacines of Australia that have been kept in aviaries (*5*,*6*). Diagnosis in all these cases was based on histopathologic detection of protozoan cyst-like structures of unexplained origin in the heart and skeletal muscles and, to a lesser extent, in other organs. In most cases, the protozoans were identified as members of the genus *Leucocytozoon* because of their morphologic features. Recent studies suggest that these cases may, in fact, have been infections of *Besnoitia* spp. (Sarcocystidae) or other unknown hematozoa (*5*); however, genetic evidence is lacking.

In August 2010, sudden deaths of parrots were noticed in 2 separate aviaries in northern Germany and Switzerland (online Technical Appendix Table, www.cdc.gov/EID/content/17/5/950-Techapp.pdf). Nine yellow-crowned parakeets (*Cyanoramphus auriceps*), 3 barred parakeets (*Bolborhynchus lineola*), and 2 budgerigars (*Melopsittacus undulatus*) died within 2–5 days after a history of reduced general conditions and reduced activity and food intake before death. In addition, 2 budgerigars and 1 barred parakeet in the aviary in Germany showed

lethargy and reduced food intake for 2 weeks but fully recovered. About half of the birds were juvenile. No new birds had been introduced into the aviaries during the previous 24 months.

Necropsy and histologic examination of 7 animals with fatal disease showed numerous large cyst-like protozoan structures (size up to 800 µm in diameter; online Technical Appendix Figure) in myocardial and skeletal muscles and, to a lesser extent, in the lung and the smooth muscles of the intestinal tract without obvious signs of inflammation. The cyst-like structures had a thick eosinophilic outer wall, were partly compartmented by internal septae, and were filled with many merozoites. Surrounding muscle fibers were degenerated or necrotic and, in some cases, associated with hemorrhage. Blood smears of clinically affected animals screened for ≈5 × 10^5 cells each did not show parasites.

To further characterize the parasites, we carried out a nested PCR and subsequent DNA sequencing as described (*7*). Notably, phylogenetic comparison of 479 bp of the mitochondrial cytochrome b gene derived from protozoan cyst-like structures with known sequences of avian hematozoa found 99%–100% homology of parasites from both outbreaks with the avian malaria parasites (*Haemoproteus* spp.) of European songbirds (Figure). Identical cytochrome b sequences were detected in a yellow-crowned parakeet from Switzerland (CYAUR1), a budgerigar from Germany (MEUND1), and a *Haemoproteus* sp. (TUPHI1) previously found in the blood of a song thrush (*Turdus philomelos*) in Bulgaria. The sequence derived from the barred parakeet (BOLIN1) of the German outbreak was identical with *H. minutus* of the common blackbird (*T. merula*). In fact, different psittacine species of the German outbreak were infected with different *Haemoproteus* spp. Because all affected parrots had been bred in Europe and had no contact to imported birds, these results suggest that infection was the result of previously unknown cross-species transmission of *Haemoproteus* spp. between birds of only distantly related orders (*8*,*9*).

Blood samples from surviving, asymptomatic animals from the German outbreak were tested cytologically and by nested PCR for the presence of *Haemoproteus* spp. PCR identified *Haemoproteus* sequences in the blood of 3 of 26 psittacines, although parasitic structures were not identifiable in blood smears. Retrieved sequences were identical with that of MEUND1, except for a single-nucleotide polymorphism in 1 sequence (MEUND3; Figure). A latent infection of these animals therefore seems possible and may constitute a potential risk for further horizontal transmission in aviaries by blood-sucking insects such as biting midges (*Culicoides*), the vectors for *Haemoproteus* spp. of passerine birds in Europe (*2*).

In conclusion, we identified the cause of a previously unexplained lethal disease of captive parrots in Europe, induced by numerous large cyst-like megalomeronts in several organs, including the heart. Morphologically, the parasitic structures were strikingly similar to yet undetermined parasites of numerous previous outbreaks (*5*,*6*). Genetically, the parasites had 99%–100% homology to known *Haemoproteus* spp. from wild European songbirds. The avian malaria parasites identified are highly prevalent in the native songbird population but generally do not cause overt disease or death in their natural hosts. In contrast, the cases reported here suggest that these parasites that have adapted to European songbirds may cause fatal outbreaks in native psittacines of Australia, New Zealand, and South America that are raised in captivity. These findings also show that preexisting pathogens may

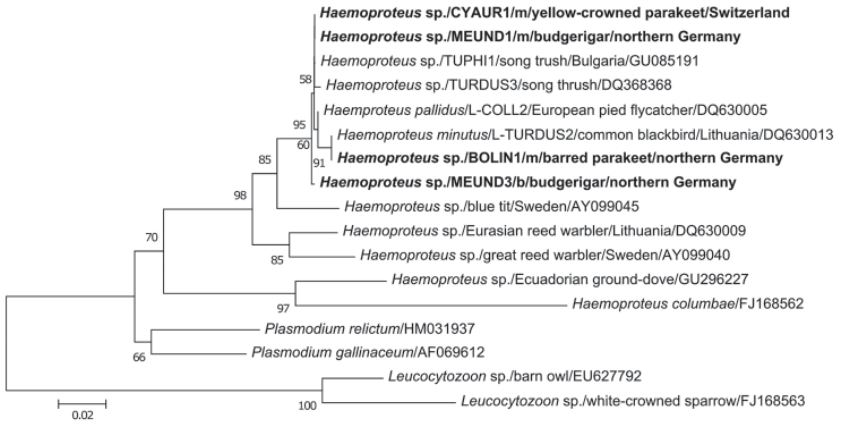

Figure. Phylogenetic relationships based on alignment of 479 bp of the cytochrome b gene of *Haemoproteus* spp. isolated from megalomeronts (m) of infected muscles and blood (b) of parrots with related hematozoan parasites in GenBank and the database MalAvi (http://mbio-serv4.mbioekol.lu.se/avianmalaria; *10*). Nucleotide distance values of the maximum likelihood phylogenetic tree were calculated under the HKY substitution model. New sequences of *Haemoproteus* spp. from parrots of this study are shown in **boldface**. Two distinct species of the genus *Leucocytozoon* served as outgroup of the phylogenetic tree. The branch lengths are proportional to the degree of inferred evolutionary change as shown by the scale bar, and the numbers indicate bootstrap values (1,000 replicates). While the cytochrome b sequences CYAUR1, MEUND1, and BOLIN1, respectively, found matching sequences, MEUND3 showed closest sequence similarities with *Haemoproteus* spp. of the lineage COLL2, which depict a wider host breadth among songbirds (http://mbio-serv4.mbioekol.lu.se/avianmalaria). The isolates of *Haemoproteus* spp. from psittacine birds were deposited into GenBank under accession nos. HQ398207–HQ398212.

be a potential hazard for invading species. Avian malaria should therefore be considered a threat for exotic parrots in Europe until results of further epidemiologic and experimental studies are available. Because many European bird species have been introduced to the native range of the psittacines studied here, a concern has been expressed that these parasites already have become established in these areas and are affecting the natural populations.

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Fatal Human Case of Western Equine Encephalitis, Uruguay

To the Editor: The genus *Alphavirus* (family *Togaviridae*) comprises 29 viral species (*1*), grouped in at least 7 antigenic complexes by their serologic cross-reactivity (*2*). They are maintained in nature through enzootic cycles involving arthropods as vectors with subsequent amplification in small mammals or birds, and epizootic cycles between mosquitoes and large mammals such as horses or humans.

Few reports have been made of the circulation of alphaviruses in Uruguay. A serologic study conducted in 1970 found antibodies to western (WEEV) and eastern equine encephalitis viruses by using complement fixation and hemagglutination inhibition tests in serum specimens from children (*3*). In 1972–1973, epizootics in horses caused by WEEV were reported in Argentina and Uruguay, and WEEV was isolated from a sick horse (*4*).

We report a fatal case of viral encephalitis in April 2009 in Montevideo, Uruguay, in a previously healthy 14-year-old boy. Four days before he sought treatment, he had fever, asthenia, and headaches. At hospital admission (April 12, 2009), he was febrile and without neurologic signs; amoxicillin treatment was initiated. Results of a computed tomography scan of the brain were normal.

On day 1, headache, vomiting, neck stiffness, and partial left seizures on the left side developed. Also exhibited were consciousness depression (Glasgow Coma Scale 12 points), hyperreflexia, and bilateral Babinski sign. A cerebrospinal fluid (CSF) sample was negative for bacteria in cultures. An electroencephalogram showed diffuse brain suffering. The patient was brought to the intensive care unit with a clinical diagnosis of viral encephalitis. Over the next 24–36 hours, intracranial hypertension developed, and medical treatment was given (sedation, hyperventilation, mannitol, and barbiturates). Conscience depression progressed to a deeper level, and a computed tomography scan of the brain showed dilatation of the temporal ventricles and compression of the peritroncal and sylvian cisterns. During the next 48 hours, the coma level went deeper, reaching 6 on the Glasgow Scale. Another CSF specimen was taken, and PCR results were negative for herpesvirus and enterovirus. Glasgow Coma Scale level was 3 on April 15, and a decompressive craniectomy was done. Seventy-two hours after admission, the patient died.

The patient's plasma and CSF were tested for antibodies to dengue and West Nile viruses (immunoglobulin M and G) through ELISA (Focus Technologies, Cypress, CA, USA) and for St. Louis encephalitis and dengue virus by M antibody-capture–ELISA (*5*). RNA was extracted from plasma and CSF, followed by a generic nested reverse transcription–PCR (RT-PCR) for flaviviruses (*6*). Serologic and molecular test results were negative for the above-mentioned pathogens. Then we performed a generic nested RT-PCR (*7*), which amplifies 448 bp at first round and 195 bp (second round) of the alphavirus NSP4 gene. Also, a heminested PCR was done (products 372 bp and 303 bp); RNA from Venezuelan equine encephalitis virus Tc-83 (provided by M. Contigiani, Universidad de Córdoba) was used as positive control. The target region is informative enough to allow the precise identification of the most relevant alphaviruses by sequencing and phylogenetic analysis. Alphavirus genome amplification was achieved for the CSF specimen collected at admission to the hospital. Plasma and a second CSF specimen were PCR negative. To confirm these findings, another nested RT-PCR reaction targeting the NSP1 gene was done as described previously (*8*). A 208-bp amplicon, which corresponded to the expected size for WEEV, was obtained from plasma and the first CSF specimen.

Sequence analyses were conducted on the NSP4 fragments. Maximum likelihood (*9*) and Bayesian (*10*) phylogenetic analyses gave similar results. The Figure, panel A, shows that sample LCR/09-303 is part of a well-supported clade (aLRT = 0.99), which groups WEEVs. The sequence LCR/09-303 is a sister taxon to sequences GQ287641 and GQ287642, with poor support (Figure, panel B) (online Appendix Table, www.cdc.gov/EID/content/17/5/952-appT.htm). These are reference WEEV USA strains (Imperial and Kern) obtained from *Culex tarsalis* mosquitoes. Our sample and the mentioned sequences are part of a well-supported clade (aLRT = 0.85), together with GQ287645, AF214040, and FJ786260. These are also USA strains; 2 were isolated from infected

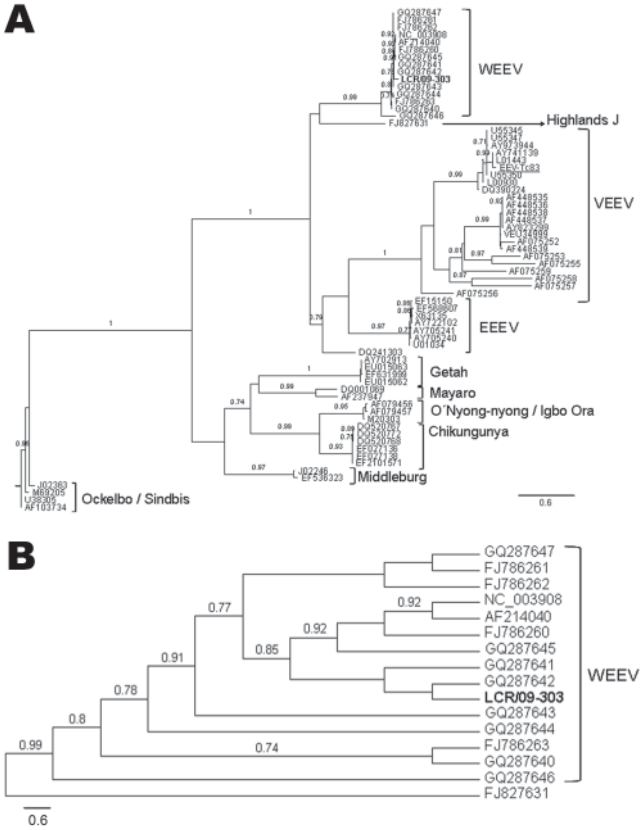

Figure. A) Phylogenetic tree obtained by maximum likelihood analysis of sequences corresponding to the alphavirus NSP4 gene. Alignment used in the analysis had 448 bp and was conducted by using BioEdit software version 7.0.9.0 (www.mbio.ncsu.edu/BioEdit/BioEdit.html). Estimation of the suitable model of nucleotide substitution was carried out by using Modelgenerator (http://bioinf.may.ie/software/modelgenerator). Phylogenetic analysis was run on the PhyML web server (www.atgc-montpellier.fr/phyml), with the following settings: nucleotide substitution model: general time reversible + proportion invariant + Γ; proportion of invariable sites: 0.39; gamma distribution parameter α: 0.67; node support: approximate likelihood-ratio test (only values over 0.70 are shown). Sequences included in the analysis were the following (GenBank accession numbers for individual isolates provided when applicable): human encephalitis case-patient: LCR/09-303 (**boldface**); reverse transcription–PCR positive control Venezuelan equine encephalitis virus [VEEV] Tc83 (282 nt), FJ786261; western equine encephalitis virus (WEEV): AF214040, GQ287647, GQ287646, GQ287645, GQ287644, GQ287643, GQ287642, GQ287641, GQ287640, FJ786263, FJ786262, FJ786260, NC003908; Highlands J virus, FJ827831; Venezuelan equine encephalitis virus (VEEV), L01443, DQ390224, AF075255, AY823299, AF448539, AF448538, AF448537, AF448536, AF448535, AF075252, U34999, AF075259, AF075256, AF075253, AF075257, AF075258, AY973944. L00930, AY741139, U55350, U55347, U55345; eastern equine encephalitis virus (EEEV), AY722102, U01034, EF568607, EF15150, AY705241, AY705240, X63135, DQ241303; Getah virus, EU015063, EU015062, EF631999, AY702913; Mayaro virus, AF237947, DQ001069; M20303; O'nyong-nyong virus, AF079456; Igbo Ora virus, AF079457; chikungunya virus, EF210157, EF027138, EF027136, DQ520772, DQ520768, DQ520767; Middleburg virus, EF536323, J02246; Ockelbo virus, M69205: Sindbis virus, AF103734, U38305, J02363, M69205. B) Detail of the WEEV clade, showing the relationships between the sample LCR/09-303 and the rest of the WEEV isolates included in the analysis. Scale bars indicate expected nucleotide changes per site.

horses and 1 from *Cx. tarsalis* mosquitoes. Notably, our sequence is distantly related to GQ287646, which was isolated from *Culex* spp. mosquitoes in Chaco, Argentina. The nucleotide sequence of the positive control VEEV-Tc83 is correctly placed in the VEEV clade.

Clinical and laboratory findings showed that the illness described here was compatible with viral encephalitis. Using a generic RT-PCR assay on an early CSF sample, we amplified a partial sequence (NSP4 gene) of an alphavirus. Phylogenetic analyses showed that the patient's sequence grouped with sequences from WEEV, with high statistical support. A second RT-PCR assay on the NSP1 gene enabled us to obtain an amplification of 208 bp, which is consistent with the expected size for WEEV. Therefore, we concluded that the fatal disease was likely caused by WEEV. Since the 1970s, to our knowledge, the presence of WEEV (or other alphaviruses) in Uruguay has not been documented. Moreover, no recent reports have been made of genome detection of WEEV in encephalitis cases in the region.

Although the case described here may be rare, the etiology of many viral encephalitides in Uruguay remains unknown. Serologic studies in horses and studies to detect arboviruses in mosquito populations are being conducted to investigate the status of arbovirus infections in Uruguay.

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Widespread Availability of Artemisinin Monotherapy in the United States

To the Editor: Artemisinin-based combination therapies are recommended as first line treatments for *Plasmodium falciparum* malaria in most areas of the world. The article by Shahinas et al. (*1*) describes a patient who had *P. falciparum* malaria after returning from Nigeria. Her isolate had an elevated 50% inhibitory concentration to artemisinin derivatives. She had obtained artesunate in Nigeria and took it weekly for malaria prophylaxis, which might have contributed to the relative resistance found.

In 2009, one artemisinin-based combination therapy (artemether/lumefantrine) became available for use in the United States. However, it is not widely appreciated that artemisinin is actually available in the United States as an herbal supplement for over-the-counter purchase (*2*). It is marketed for general health maintenance and for treatment of parasitic infections and cancers (Figure), although as with other supplements it is not intended to diagnose, treat, cure, or prevent any disease. As in the patient described by Shahinas et al., widespread use

Figure. Bottle of artemisinin, available over-the-counter as an herbal supplement.

of artemisinin or its derivatives as monotherapies could potentially lead to progressively increasing resistance in *P. falciparum* malaria (*3*). Studies in western Cambodia, where artemisinin monotherapy has been available for many years, have revealed in vivo artesunate resistance, with markedly decreased parasite clearance times (*3*). Progressive spread of artemisinin resistance could have disastrous consequences for the global control of malaria. Thus, minimally regulated use of potent compounds in dietary supplements has the potential for major public health implications.

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Yersinia pestis DNA Sequences in Late Medieval Skeletal Finds, Bavaria

To the Editor: We read with interest the report by Wiechmann et al. that, in the investigation of late medieval plague, partial sequencing of the *Yersinia pestis* pPCP1 plasmid yielded the observation of a 3-T homopolymeric tract which differed from the 5-T homopolymeric tract of the Orientalis *Y. pestis* CO92 type strain (*1*). This observation was unexpected because previous data from multispacer sequence typing and *glp* D gene sequencing yielded only the Orientalis biotype in cases of ancient plague (*2*).

Using suicide PCR (*3*), we therefore further investigated pPCP1 in 10 negative control dental pulp specimens and 60 specimens collected from 1 Justinian Orientalis plague site (*2*), 2 Black Death Orientalis sites, and 2 additional medieval plague sites. All negative controls remained negative; 14 (23%) of 60 plague specimens yielded a PCR product, and 7 interpretable sequences yielded a 3-T homopolymeric tract in all cases.

We further tested a *Y. pestis* isolate collection comprising 2 Antiqua, 6 Medievalis, and 4 Orientalis strains. No amplification was obtained in DNA-free PCR mix and 5 *Y. enterocolitica*–negative control isolates, whereas sequencing yielded a 3-T homopolymeric tract in all 12 *Y. pestis* isolates.

BLAST analysis (http://blast.ncbi.nlm.nih.gov/blast.cgi) indicated that the 5-T homopolymeric tract has been found only once in the *Y. pestis* CO92 strain (*4*) and in none of 22 modern and 11 ancient sequences (Table). This 5-T homopolymeric tract is therefore CO92 strain specific and not a marker for the Orientalis biotype. This pPCP1 plasmid sequence, located into a noncoding region of the 3′ extremity of the plasmid, is characterized by several homopolymeric tracts of poly (A) and poly (T), including the 1 herein investigated. Instability of the T-stretches has been reported in bacterial genomes (*5*) as being hot spots for mutations (*5*).

Therefore, in our assessment, the data reported for the late medieval Bavaria burial (*1*) do not support that deaths of persons buried in this site resulted from a non-Orientalis plague. Typing modern or ancient *Y. pestis* strains should not rely on poly (A) and poly (T) homopolymeric tracts sequencing.

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Table. Alignment of pPCP1 *Yersinis pestis* modern and ancient sequences

Source and *Y. pestis* strain	GenBank accession no.	Sequence, 5′ → 3′
Complete sequence		
Y. pestis CO92 plasmid pPCP1	AL109969.1	8488_TATATTTTCAAGAAAAGCTGGCTATTTAACAT AACGGCAATTTTTGTACGCACCACTGAA_8547
Y. pestis KIM plasmid pPCP1	AF053945.1	8488_TATATTTTCAAGAAAAGCTGGCTATTTAACAT AACGGCAATTT..GTACGCACCACTGAAAT_8547
Y. pestis biovar Microtus str. 91001 plasmid pPCP1	AE017046.1	8487_TATATTTTCAAGAAAAGCTGGCTATTTAACAT AACGGCAATTT..GTACGCACCACTGAAAT_8546
Y. pestis Nepal516 plasmid pPCP	CP000307.1	9650_TATATTTTCAAGAAAAGCTGGCTATTTAACAT AACGGCAATTT..GTACGCACCACTGAAAT_9709
Y. pestis Antiqua plasmid pPCP	CP000310.1	9661_TATATTTTCAAGAAAAGCTGGCTATTTAACAT AACGGCAATTT..GTACGCACCACTGAAAT_9720
Y. pestis D182038 plasmid pPCP1	CP001592.1	8486_TATATTTTCAAGAAAAGCTGGCTATTTAACAT AACGGCAATTT..GTACGCACCACTGAAAT_8545
Y. pestis Z176003 plasmid pPCP1	CP001596.1	8487_TATATTTTCAAGAAAAGCTGGCTATTTAACAT AACGGCAATTT..GTACGCACCACTGAAAT_8546
Modern isolate		
103813 *Y. pestis* Nairobi rattus Antiqua	HQ542863	61_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAAT_120
103814 *Y. pestis* JHUPRI Antiqua	HQ542864	61_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAAT_120
103815 *Y. pestis* 14–47 Medievalis	HQ542865	61_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAAT_120
103817 *Y. pestis* 5G5 Medievalis	HQ542866	61_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAAT_120
103818 *Y. pestis* 5F1 Medievalis	HQ542867	61_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAAT_120
103819 *Y. pestis* 6B4 Medievalis	HQ542868	61_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAAT_120
103820 *Y. pestis* 8B7 Medievalis	HQ542869	61_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAAT_120
103821 *Y. pestis* 9F11 Medievalis	HQ542870	61_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAAT_120
103822 *Y. pestis* 6/69M Orientalis	HQ542871	61_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAAT_120
103823 *Y. pestis* EV-76 Orientalis	HQ542872	61_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCAC_114
103824 *Y. pestis* algeria 1 Orientalis	HQ542873	64_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAA_123
103825 *Y. pestis* algeria 2 Orientalis	HQ542874	61_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAAT_120
Ancient strain detected from teeth		
Tooth no. 107 (excavated from Lariey site, France, 17th century)	HQ542875	62_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAATGC_123
Tooth no. 515 (excavated from Venice site, Italy, 14th–16th centuries)	HQ542876	60_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAATGC_121
Tooth no.1183 (excavated from Bondy site, France, 11th–15th centuries)	HQ542877	62_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAAT_121
Tooth no. 1184 (excavated from Bondy site, France, 11th–15th centuries)	HQ542878	61_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAAT_121
Tooth no. 1190 (excavated from Bondy site, France, 11th–15th centuries)	HQ542879	61_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAAT_120
Tooth no. 254 (excavated from Venice site, Italy, 14th–16th centuries)	HQ542880	61_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAAT_120
Tooth no. 1180 (excavated from Bondy site, France, 11th–15th centuries)	HQ542881	61_TATATTTTCAAGAAAAGCTGGCTATTTAACATAA CGGCAATTT..GTACGCACCACTGAAAT_120

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In Response: We thank Tran et al. for their interest in our article (*1*). In it, we described the detection of several *Yersinia pestis*–specific plasmid pPCP1 DNA sequences in skeletal remains from a late medieval mass burial in Bavaria, Germany. In 1 of these sequence sections, we found a deviation from the reference sequence used (*Y. pestis* strain CO92 plasmid sequence AL109969.1). We did not further interpret this result because we agree with Tran et al. that typing of *Y. pestis* strains should not rely on poly (A) and poly (T) homopolymeric tract sequencing (*2*). As we have stated (*1*), further analyses of our material, including chromosomal markers (*3*,*4*) will be conducted to obtain clues as to the specific *Y. pestis* strain.

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Stelios Faitakis (b. 1976) *Kakerlaken sind die Zukunft* (2009) Mixed media on canvas (260 cm × 190 cm). Courtesy of The Breeder gallery@thebreedersystem.com, Athens. Photo: Vivianna Athanasopoulou

And therefore I have sailed the seas and come To the holy city of Byzantium

—William Butler Yeats

Polyxeni Potter

"One morning, as Gregor Samsa was waking up from anxious dreams, he discovered that in his bed he had been changed into a monstrous verminous bug," wrote Franz Kafka in The Metamorphosis. "He lay on his armor-hard back and saw, as he lifted his head up a little, his brown, arched abdomen divided up into rigid bow-like sections. From this height the blanket, just about ready to slide off completely, could hardly stay in place. His numerous legs, pitifully thin in comparison to the rest of his circumference, flickered helplessly before his eyes."

Kafka's nightmarish tale captures the essence of unexpected uncontrollable life-defining horror, likely caused by Gregor Samsa's inability to cope with societal and family pressures. His predicament, much like any severe disfiguring and disabling illness, would isolate and eventually kill him. Transformative experiences have been the domain of poets and artists alike because artistic sensibility heightens awareness of reality, prompting them to seek a better or more comprehensible alternative. Such seems to be the goal of Stelios Faitakis, who from his native Greece has set out to understand and convey to all a meaningful version of the world around him.

"I was raised in the west suburbs of Athens," recounted Faitakis in a 2007 interview, "a place occupied mostly by the working class." A serious injury during childbirth left him with substantial paralysis of the right arm and, despite extensive surgical and other interventions, it would limit his activities, including painting, to his left arm. "Both my parents were workers in a gold chain factory…. They are not what we'd call 'artists,' although when I see my mother creating new designs… my artistic nature has some root there…. Also my grandfather… was a good draftsman…. I have painted since childhood."

Overcoming family opposition, Faitakis found his way into the prestigious Academy of Fine Arts in Athens, where he set out on his artistic journey, guided as much by his affinity to mathematics as by Eastern mysticism. Suspicious of authority and large organized institutions, he is self-reliant and openly critical of political oppression. "As long as I can remember, I've been an anarchist." He views art as an inclusive and enlightening agent. His first efforts to reach the public came as graffiti—in the streets of his hometown and later on the walls of the Academy. "Working for the public for me means mostly painting

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outdoors in the open, in the streets, where everyday people pass to go to their jobs…. Athens… begs to be painted." His murals have now dotted the globe, from European cities to Miami's Wynwood Art District.

"From the beginning, I chose to paint narrative pictures, like a still from a theatrical play: human characters in some environment doing some action—the simplest scenario possible," with hidden meanings, "as an extra for the more demanding eyes." His heavily populated canvases and murals of common folk at work and play have been likened to the paintings of Pieter Bruegel the Elder; Mexican muralism, a monumental form of wall painting accessible to the masses; Japanese screen painting; and the Cretan School, whose style of icon painting, also seen in the work of El Greco, flourished during the late Middle Ages and peaked after the fall of Constantinople, becoming a major force in Greek painting during the 15th through the 17th centuries.

But what has brought Faitakis into his own is a consistent reference to art forms rooted in the Byzantine tradition. "It would never be possible for me to neglect this element…. I paint about Humanity and its relation to itself… so my characters flow in a golden world…. Simple, ordinary colors coexist with metallic/light reflecting colors…. The gold refers to eternity, universal time." The tradition relies on exegesis, "Art should be used as a tool for human beings to… grow." In Faitakis' work, monastic settings, precipitous mountains, and hermetic deserts unite with urban scenes, political unrest, and common ailments to seek resolution in unorthodox ways. "Art opens the human being to the use of capabilities that our modern civilization has shrunk, such as intuition and inspiration."

In *Kakerlaken sind die Zukunft*, on this month's cover, repetitive detailed patterns and elements from psychology, the natural sciences, popular wisdom, and political allegory unite with humor and drama to create aggressive commentary. Perspective is achieved by stacking objects on top of each other. Buildings, decaying cities and surroundings, complex geometric and floral designs, and message ribbons portray, without physical barriers or reference to place and time, the universe. Size denotes importance, so the insect containing the urban scene, placed in full frontal position and regalia, spells an ominous message: vermin can outnumber, outdo, and outlive the human community. Larger than life and wearing a person's head, this all-weather vector feeds on poverty, ignorance, disease, and death.

Frustrated by the state of affairs, the painter abandons the mundane and, like W.B. Yeats, sails to Byzantium—not a destination but an idea. For as the poet says, "the only way for the soul to learn to sing is to study "monuments of its own magnificence." In the vernacular of a bygone culture, he transcends reality to transform obscure troublesome prospects to legible content accessible to all. As the Kafkaesque scenario unfolds, modern inventions move in and out the stylized landscape—a huge electronic screen; helicopters buzzing like giant insects with petal-like rotors, steam haloes, and landing limbs. A tiny human lurks in a shadowy crevice on the side. Beyond exegesis, this eerie scene invites anagogical interpretation.

Much like a scientist with a microscope, Faitakis amplifies figures and their surroundings to take a closer look at vermin and force the viewer to experience their presence and resilience. But in painting this anthropomorphic insect, he also creates an icon of the ubiquitous vector, which transmits viruses, bacteria, and other pathogens, among and between humans and animals, forming a bridge over spatial, behavioral, and ecologic barriers and promoting the emergence of disease. Yellow fever virus is transmitted from monkeys to humans by mosquitoes; Lyme disease, from rodents to humans by ticks. And though Faitakis' insect contains the city, a vector is not just a vessel. Always evolving as part of the elaborate transmission mechanism, a vector provides the pathogen itself ways to evolve, creating even more opportunities for disease emergence.

The complexity of human-animal-vector interactions underlying *Kakerlaken sind die Zukunft* adds another dimension to Faitakis' surreal universe. And like the sociopolitical elements, these interactions are staggering and require deeper understanding that may reside "as in the gold mosaic of a wall" at the convergence of human behavior, vector biology, climate, and land use.

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To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions and earn continuing medical education (CME) credit, please go to **www.medscape.org/journal/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit is acceptable as evidence of participation in CME activities. If you are not licensed in the US and want to obtain an AMA PRA CME credit, please complete the questions online, print the certificate and present it to your national medical association.

Article Title
Lessons Learned about Pneumonic Plague Diagnosis from 2 Outbreaks, Democratic Republic of the Congo

CME Questions

1. You are a public health official asked to consult in a war-torn region of Africa nonendemic for pneumonic plague. Based on the above report by Dr. Bertherat and colleagues, which of the following observations concerning a new outbreak of respiratory infection would make pneumonic plague most likely?

A. Productive cough
B. Gradual progression of symptoms over 1-2 weeks
C. Spontaneous recovery in most cases
D. High mortality rate within 2–4 days after respiratory exposure

2. Based on the above report, which of the following statements about recent outbreaks of pneumonic plague in the Democratic Republic of the Congo (DRC) is most likely correct?

A. Diagnostic strategies used for the 2005 and 2006 outbreaks were similar
B. The Oriental Province where the outbreaks occurred had pre-existing stable infrastructure facilitating diagnosis
C. In the second outbreak, detecting the F1-antigen by rapid diagnostic tests (RDT) strengthened the frontline response strategy
D. Specialized sampling kits allowed rapid recognition of the first outbreak

3. As a public health official (described in Question 1), which of the following are you most likely to consider for frontline response strategy to pneumonic plague based on the above report?

A. Transporting specimens to distant laboratories with better facilities is preferred
B. Specimen collection kits and RDT must be provided for first-line detection and collection
C. RDT is sufficient for surveillance
D. Outbreaks in previously plague-free areas can be managed locally

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Article Title
Severe Imported *Plasmodium falciparum* Malaria, France, 1996–2003

CME Questions

1. You are seeing a 30-year-old man with fever for 5 days. He returned from a trip to Kenya 3 days ago. Which of the following statements regarding malaria among travelers is most accurate?

A. Malaria is the most common cause of fever among travelers returning from the developing world
B. Most cases of malaria among returning travelers are classified as severe
C. Death due to malaria, even severe malaria, is extremely rare among returning travelers
D. Adults from endemic areas are at the highest risk for death from malaria acquired during travel to areas with a high prevalence of malaria

2. You suspect malaria in the patient described in question 1. What were characteristics of patients with malaria in the current study?

A. Malaria was most common among European travelers
B. Most patients had received appropriate chemoprophylaxis
C. Most patients had symptoms before returning from travel
D. 75% of patients were hospitalized

3. The patient in question 1 is diagnosed with malaria. Which of the following demographic and travel variables was most associated with a higher risk for severe malaria in the current study?

A. Male sex
B. African origin
C. Travel to East Africa
D. Lower socioeconomic status

4. Which of the following medical variables was most associated with a higher risk for severe malaria in the current study?

A. Thrombocytopenia
B. Initial presentation to a hospital
C. Absence of chemoprophylaxis
D. Normal serum leukocyte count

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Laboratory Service Effectiveness during Pandemic (H1N1) 2009, Australia

Wild Birds and Transmission of Highly Pathogenic Avian Influenza (H5N1) among Poultry Flocks, Thailand

Pandemic (H1N1) 2009 Risk for Frontline Health Care Workers

Binary Toxin and Death after *Clostridium difficile* Infection

Invasive Group A Streptococcal Infection, Auckland, New Zealand

Methicillin-Resistant *Staphylococcus aureus*, Samoa

Surveillance and Screening for *Taenia solium* Infection, Oregon, USA

Cefepime-Resistant *Pseudomonas aeruginosa*

Effectiveness of Antiviral Drugs in Reducing Household Transmission of Pandemic (H1N1) 2009

Marked Campylobacteriosis Decline after Interventions Aimed at Poultry Processing, New Zealand

Multiple Introductions of Multidrug-Resistant Tuberculosis into Households, Lima, Peru

Reflections on 30 Years of AIDS

Catheter-related Bacteremia Caused by Methicillin-Susceptible *Staphylococcus sureus*

Salmonella enterica Serotype Typhi and Nonclassical Quinolone Resistance Phenotype

Determinants of Serologic Failure after Treatment for Yaws

Severe Leptospirosis with Clinical Signs Similar to Pandemic (H1N1) 2009

Ciprofloxacin-Resistant *Salmonella enterica* Serotype Typhi Isolates in the United States, 1999–2008

Complete list of articles in the June issue at
http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

July 8–10, 2011
International Society for Infectious Diseases Neglected Tropical Diseases Meeting (ISID-NTD)
Boston, MA, USA
http://ntd.isid.org

August 8–19, 2011
12th International Dengue Course
Havana, Cuba
http://www.ipk.sld.cu/cursos/dengue2011/index.htm

August 27–31, 2011
2011 Infectious Disease Board Review Course – 16th Annual Comprehensive Review for Board Preparation
Ritz-Carlton, Tysons Corner
McLean, VA, USA
http://www.IDBoardReview.com

September 17–20, 2011
51st Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC)
McCormick Place Chicago
Chicago, IL, USA
http://www.icaac.org

October 12–15, 2011
The Denver TB Course
Denver, CO, USA
http://www.njhealth.org/TBCourse

October 20–23, 2011
49th Annual Meeting of the Infectious Diseases Society of America
Boston, MA, USA
http://www.idsociety.org/idsa2011.htm

November 16–19, 2011
7th World Congress of the World Society for Pediatric Infectious Diseases (WSPID 2011)
Melbourne, Australia
http://www.kenes.com/wspid2011/mailshot/ms3.htm

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). In 50–150 words, describe timely events of interest to our readers. Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

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

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post some articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (www.cdc.gov/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit figures as separate files, in the native format when possible (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpg or .tif files. Other file formats may be acceptable; contact fue7@cdc.gov for guidance. Figures should not be embedded in the manuscript file. Use color only as needed. Use Arial font for figure lettering. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced to print size. Large figures may be made available online only. Place figure keys within the figure; figure legends should be provided at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may 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.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and 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 biographical sketch. Dispatches are updates on infectious disease trends and research that 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.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

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. Include biographical sketch.

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. No biographical sketch is needed.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only and should contain 500–1,000 words. They should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.