# EMERGING INFECTIOUS DISEASES®

June 2012

## Prion Diseases

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June 2012

## On the Cover

**Gerard van Kuijl,**
**Dutch painter active in Rome (1604–1673)**
***Narcissus* (c. 1645)**
**Oil on canvas**
**(114 cm × 143 cm)**

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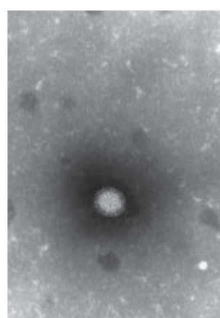

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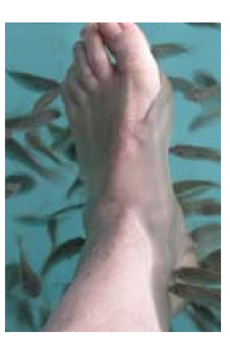

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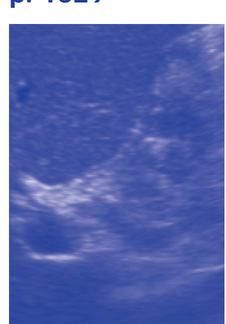

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# Iatrogenic Creutzfeldt-Jakob Disease, Final Assessment

**Paul Brown, Jean-Philippe Brandel, Takeshi Sato, Yosikazu Nakamura, Jan MacKenzie, Robert G. Will, Anna Ladogana, Maurizio Pocchiari, Ellen W. Leschek, and Lawrence B. Schonberger**

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**Release date: May 16, 2012; Expiration date: May 16, 2013**

**Learning Objectives**

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

- Distinguish the principal sources of iatrogenic CJD
- Identify countries with the highest rates of documented CJD
- Analyze the clinical presentation of iatrogenic CJD
- Assess new threats which might promote higher rates of CJD.

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The era of iatrogenic Creutzfeldt-Jakob disease (CJD) has nearly closed; only occasional cases with exceptionally long incubation periods are still appearing. The principal sources of these outbreaks are contaminated growth hormone (226 cases) and dura mater grafts (228 cases) derived from human cadavers with undiagnosed CJD infections; a small number of additional cases are caused by neurosurgical instrument contamination, corneal grafts, gonadotrophic hormone, and secondary infection with variant CJD transmitted by transfusion of blood products. No new sources of disease have been identified, and current practices, which combine improved recognition of potentially infected persons with new disinfection methods for fragile surgical instruments and biological products, should continue to minimize the risk for iatrogenic disease until a blood screening test for the detection of preclinical infection is validated for human use.

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The first case of what would eventually become a major outbreak of iatrogenic Creutzfeldt-Jakob disease (CJD) was reported in 1974; the patient had received a corneal transplant from an infected cadaver (*1*). In the years that followed, other sources of infection were identified: stereotactic electroencephalogram electrodes, neurosurgical instruments, cadaveric dura mater and pituitary glands, and, most recently, secondary variant CJD (vCJD) blood products. The ensemble of iatrogenic cases, including a bibliography of primary references, was last reviewed in 2006 (*2*). Today, after nearly 40 years of surveillance, the chronology and essential characteristics of iatrogenic CJD have been finalized, and the purpose of this article is to present these data along with a few brief comments about factors that determined the risk for infection and how future risks might be foreseen and avoided.

By far the most common sources of iatrogenic disease were human cadavers from which pituitary hormones and dura mater grafts were obtained (Table 1; Figure); the other major variety of environmentally acquired disease is vCJD. The incidence curves of human growth hormone–associated and dura mater–associated CJD are almost superimposable; a broad peak occurred in the mid-to-late 1990s, just ahead of the sharper peak incidence of vCJD in the United Kingdom at the turn of the century. The incidence in other countries peaked a few years later, in 2004, as a result of the delayed appearance of bovine spongiform encephalopathy in those countries.

The long incubation periods—years to decades—of these low-dose infections pose a particularly difficult problem for public health officials, whose recommendations may diminish the number of new cases but are impotent when it comes to preventing cases in already-infected persons in the preclinical phase of disease. It is worth remembering that the early recognition of iatrogenic sources of CJD was entirely because of a few remarkably astute neurologists, neurosurgeons, and, astonishingly, a pediatric endocrinologist who pursued the unlikely (and unpopular) diagnosis of CJD in a growth hormone recipient (*3*). It is true that some of these connections had the benefit of comparatively short intervals between the infecting events and the onset of CJD. It is especially fortunate from the standpoint of early recognition of the dura mater association that the interval of 19 months between the operation and onset of symptoms in the first case-patient was among the shortest on record for this form of iatrogenic CJD (Table 2).

## Human Growth Hormone

The current worldwide total of growth hormone–associated cases of CJD is 226. Most cases occurred in France (119 cases/1,880 recipients; attack rate 6.3%), the United Kingdom (65 cases/1,800 recipients; attack rate

Table 1. Global distribution of cases of iatrogenic Creutzfeldt-Jakob disease*

| | Source of infection and no. cases | | | | | | |
|---|---|---|---|---|---|---|---|
| | Surgical procedure | | | | Medical procedure | | |
| Country | Dura mater grafts | Surgical instruments | EEG needles | Corneal transplants† | Growth hormone‡ | Gonadotropin | Packed red blood cells§ |
| Argentina | 1 | | | | | | |
| Australia | 5 | | | | | 4 | |
| Austria | 3 | | | | 1 | | |
| Brazil | | | | | 2 | | |
| Canada | 4 | | | | | | |
| Croatia | 1 | | | | | | |
| France | 13 | 1 | | | 119 | | |
| Germany | 10 | | | 1 | | | |
| Ireland | | | | | 1 | | |
| Italy | 9 | | | | | | |
| Japan | 142 | | | | | | |
| Netherlands | 5 | | | | 2 | | |
| New Zealand | 2 | | | | 6 | | |
| South Korea | 2 | | | | | | |
| Qatar | | | | | 1 | | |
| South Africa | 1 | | | | | | |
| Spain | 14 | | | | | | |
| Switzerland | 3 | | 2 | | | | |
| Thailand | 1 | | | | | | |
| United Kingdom | 8 | 3 | | | 65 | | 3 |
| United States | 4 | | | 1 | 29 | | |
| Total | 228 | 4 | 2 | 2 | 226 | 4 | 3 |

*EEG, electroencephalogram.
†Additional possible single cases after corneal transplant or keratoplasty (not included in table) in Japan, United Kingdom, and United States.
‡Human growth hormone given in Brazil and New Zealand was prepared in the United States; that given in Qatar was prepared in France. Additional possible single cases with human growth hormone as source (not included in table) occurred in Sweden, Australia, and New Zealand.
§An additional asymptomatic but infected red-cell recipient died of an unrelated illness; another asymptomatic infected hemophilia patient who had been exposed to potentially contaminated factor VIII also died of an unrelated illness (neither is included in the table).

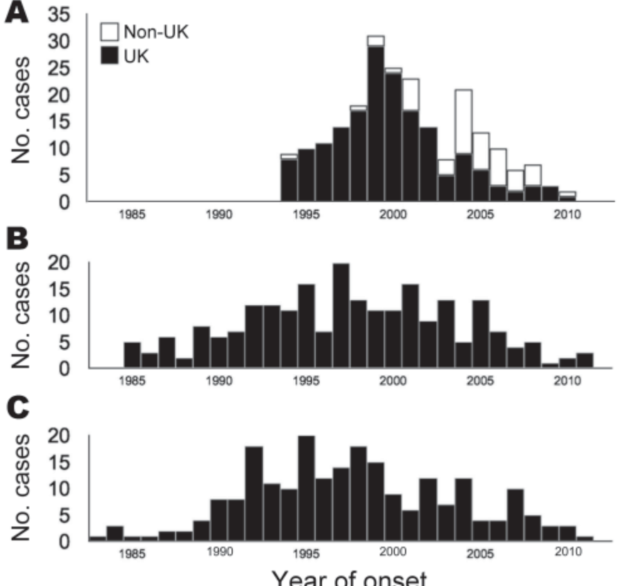


Figure. Annual incidence of variant Creutzfeldt-Jakob disease (vCJD) caused by ingestion of meat products contaminated with bovine spongiform encephalopathy agent (A) and iatrogenic CJD caused by contaminated dura mater (B) and cadaveric human growth hormone (C), 1982–2011. White bars in panel A represent cases from outside the United Kingdom, which were delayed in parallel with the later appearance of bovine spongiform encephalopathy outside the United Kingdom (not a second wave resulting from codon 129 genotype differences). Two patients are excluded: 1 presymptomatic patient from the United States who received human growth hormone and died of an intercurrent illness and 1 dura mater recipient from the United Kingdom with disease onset in 1978.

3.6%), and the United States (29 cases/7,700 recipients; attack rate 0.4%).

In France, further epidemiologic observations have revealed that all 119 cases occurred within a 1,170-patient cohort receiving treatment during a 20-month period, from December 1983 through July 1985, when there seems to have been substantial contamination resulting from sourcing and processing deficiencies. According to these numbers, the attack rate for the at-risk cohort in France increases to 10.2%. No new case has been identified since 2008. In the United Kingdom, no cohort pattern is evident, and cases continue to occur at an average rate of about 2 per year (only 1 in 2011). In the United States, CJD has not occurred in any patient who started treatment after 1977, when a highly selective column chromatography step was introduced into the purification protocol. Since 2003, only 2 new cases have been identified (1 in 2007 and 1 in 2009). An estimated ≈2,700 patients received treatment before 1977, so the attack rate in the United States for this at-risk cohort increases to 1.1% (*4*). The revised attack rates therefore become 10.2% in France, 3.6% in the United Kingdom, and 1.1% in the United States.

The methionine (M)/valine polymorphism at codon 129 of the *PRNP* gene has been examined in populations with and without CJD in many countries; results have varied (Table 3). Overall, it is clear that the M allele bestows substantial susceptibility to the sporadic and the iatrogenic forms of CJD; in consequence, the proportion of persons with MM homozygous genotype is overrepresented in both categories of disease (the sole exception occurred in UK growth hormone recipients, which led to speculation that a different strain of the pathogenic agent might have been disseminated) (*10*). It is also clear that, as a group, persons with heterozygous genotype had longer incubation periods than did those with homozygous genotype, particularly in France. Notwithstanding this statistical conclusion, it is noteworthy that several persons with MM homozygous genotype had incubation periods >30 years, including a patient with recently diagnosed CJD, whose incubation period was 42 years, the current world record for any type of iatrogenic disease.

Incubation periods for the total case population (not just those examined for the codon 129 genotype) ranged from 5 to 42 years (mean 17 years), based on the interval between the midpoint date of what was almost always a multiyear period of treatment and the onset of CJD symptoms; the actual date of infection is impossible to determine. Mean incubation periods for cases in the United States and New Zealand (patients received hormone made in the United States) were 22 and 26 years; United Kingdom, 20 years; and France, 13 years. The shorter incubation periods in France could have resulted partly from the narrower limit for the date of infection in France and are in accord with the mean incubation period of 13.5 years in the 4 gonadotropin recipients from Australia, for whom there is an even more precise date of infection. However, a greater contribution probably came from different infectious doses received by patients in the different countries. Among all patients, the clinical features were distinctive in that, unlike sporadic CJD, signs and symptoms almost never included dementia, which, if it occurred at all, was typically a late component of the clinical course.

## Dura Mater

The worldwide tally of dura mater–associated cases is 228, and new cases still continue to occur here and there, the most recent being individual cases in Austria, South Korea, and the Netherlands in 2011. If the pharmaceutical industry (in contrast to government-sponsored laboratories) comes away from the growth hormone story with an almost untainted record—only 1 case has been attributed to industrially prepared hormone (*11*)—the same cannot be said about the private sector producing dura mater grafts. The source of almost all infections was a manufacturer in Germany, B. Braun Melsungen AG, which has a worldwide

Table 2. Incubation periods and clinical presentations of iatrogenic Creutzfeldt-Jakob disease, according to source of infection*

| Source of Infection | No. cases | Mean incubation period, y (range) | Clinical signs† |
|---|---|---|---|
| Dura mater graft | 228 | 12 (1.3–30) | Cerebellar, visual, dementia |
| Neurosurgical instruments | 4 | 1.4 (1–2.3) | Visual, dementia, cerebellar |
| Stereotactic EEG needles | 2 | 1.3, 1.7 | Dementia, cerebellar |
| Corneal transplant | 2 | 1.5, 27 | Dementia, cerebellar |
| Growth hormone | 226 | 17 (5–42)‡ | Cerebellar |
| Gonadotropin | 4 | 13.5 (12–16) | Cerebellar |
| Packed red blood cells§ | 3 | 6.5, 7.8, 8.3 | Psychiatric, sensory, dementia, cerebellar |

*EEG, electroencephalogram.
†In order of decreasing frequency.
‡Averages and ranges were 13 (5–24) y in France; 20 (7–39) y in the United Kingdom; and 22 (10–42) y in the United States.
§An additional asymptomatic but infected red-cell recipient died of an unrelated illness; another asymptomatic infected hemophilia patient who had been exposed to potentially contaminated factor VIII also died of an unrelated illness (neither is included in the table).

distribution network, and the incidence of CJD appears to have more or less paralleled the frequency with which this source of dura mater was used. In Japan, it is estimated that as many as 20,000 patches may have been used each year, and the 142 cases in that country constitute two thirds of the global total. Nevertheless, the overall attack rate in the at-risk patient population in Japan is <0.03%. For the entire (worldwide) group of dura mater–recipient patients, incubation periods ranged from 1.3 to 30 years (mean 12 years), and, except in Japan, the clinical and neuropathologic features were similar to those of sporadic CJD. In Japan, approximately one third of the cases had atypical features (slow progression, noncharacteristic electroencephalogram tracings, plaque deposition, and an atypical prion protein molecular signature on Western blots), which suggested the possibility of 2 different strains of infecting agent (*12,13*). One patient had florid plaques and a pulvinar sign on magnetic resonance imaging, mimicking vCJD (*5*).

Evaluation of the influence of the codon 129 genotype is complicated by the fact that the population in Japan, among whom most cases occurred, has a high frequency of the M allele (>90%), which dominated sporadic and dura mater–associated forms of CJD (Table 3) (*6–9,14,15*). Among the cases in persons not from Japan, the distribution of genotypes approximated that found among patients with sporadic CJD, and, as with growth hormone–associated cases, incubation periods were somewhat longer for persons with heterozygous than with homozygous genotypes.

## Current Prevention Strategies

The best way to abolish secondary iatrogenic infections is, obviously, to prevent primary infections, but without a test to identify infected but asymptomatic persons, we cannot entirely eliminate the risk inherent in human-to-human tissue transfer. We are therefore obliged to rely on the default strategies of 1) identification and donor deferral of persons at higher than normal risk for CJD development and 2) inclusion of prion-reduction steps in the sterilization of penetrating instruments and the processing of therapeutic tissues and fluids.

Delineation of high-risk categories initially focused on precisely those groups of persons who were exposed to the known sources of iatrogenic disease: recipients of cadaveric dura mater grafts or pituitary-derived hormones. When vCJD started to occur, restrictions were also placed on donor time of residence in the most heavily infected regions—the United Kingdom and, to a lesser extent, continental Europe—and embargoes were placed on the importation of biological products from these regions. These deferral and import restrictions remain in place today and need some thoughtful reevaluation in view of the near extinction of all such sources of iatrogenic CJD. In the United States, there have been only 4 cases of dura mater–associated disease (the most recent in 2005) and no case of growth hormone–associated CJD for anyone who began treatment after 1977.

On the other hand, the possibility of iatrogenic infection resulting from transfer of tissues or fluids from persons who have contracted a prion disease from animals has not disappeared with the abating epidemics of bovine spongiform encephalopathy and vCJD. A few persons who may be experiencing a long incubation phase of vCJD still pose an obvious danger in the United Kingdom, but an underappreciated potential danger lies in 2 other animal diseases: scrapie and chronic wasting disease (CWD). Although scrapie-infected sheep tissues have been consumed for long enough (hundreds of years) to be considered harmless for humans, the same cannot be said about the atypical strains of scrapie that are beginning to displace the typical strains and with which we do not yet have enough experience to evaluate human pathogenicity. Similarly, we cannot declare with certainty that CWD poses no threat to humans, and CWD is continuing its unchecked spread across the United States and Canada with no guarantee that it will not become globally distributed in the years to come. One hunter has already put a group of unwitting persons at risk for infection by donating a deer, later found to have CWD, for consumption at a rural banquet in New York State (*16*); more such exposures are likely to occur as CWD continues its geographic expansion.

Table 3. Comparison of *PRNP* codon 129 genotype frequencies and incubation periods in growth hormone– and dura mater–associated cases of iatrogenic CJD*

| Category | MM | VV | Homozygotes | Heterozygotes |
|---|---|---|---|---|
| Population | | | | |
| Healthy Caucasian, %† | 40 | 10 | 50 | 50 |
| European, with sporadic CJD, % | 67 | 17 | 84 | 16 |
| Healthy Japanese, % | 92 | 0 | 92 | 8 |
| Japanese, with sporadic CJD, (%) | 97 | 1 | 98 | 2 |
| Infection source | | | | |
| Growth hormone | | | | |
| France (111) | | | | |
| Genotype frequency, % | 54 | 15 | 69 | 31 |
| Incubation period, y | 12 | 9 | 11 | 17 |
| United Kingdom (28) | | | | |
| Genotype frequency, % | 4 | 50 | 54 | 46 |
| Incubation period, y | 21 | 18 | 20 | 23 |
| United States (11) | | | | |
| Genotype frequency, % | 55 | 18 | 73 | 27 |
| Incubation period, y | 21 | 18 | 20 | 23 |
| Combined total (150) | | | | |
| Genotype frequency, % | 45 | 22 | 67 | 33 |
| Incubation period, y | 13 | 12 | 13 | 17 |
| Dura mater | | | | |
| Japan (54)‡ | | | | |
| Genotype frequency, % | 96 | 0 | 96 | 4 |
| Incubation period, y | 16 | NA | 16 | 13 |
| Countries other than Japan (54)§ | | | | |
| Genotype frequency, % | 65 | 15 | 80 | 20 |
| Incubation period, y | 12 | 12 | 12 | 16 |
| Combined total (108) | | | | |
| Genotype frequency, % | 81 | 7 | 88 | 12 |
| Incubation period, y | 14 | 12 | 14 | 16 |

*CJD, Creutzfeldt-Jakob disease; M, methionine; V, valine; NA, not applicable. All values are rounded to the nearest whole number.
†Based on several large-scale population studies (*5*–*9*).
‡Personal communication from M. Yamama, Department of Neurology, Kanazawa University Hospital, Kanazawa, Japan.
§Cases from France (11), Spain (11), Germany (10), Italy (8), the Netherlands (5), and 1 or 2 cases from each of 6 other countries with Caucasian populations.

## Future Prevention Strategies

The issue of reducing risk by taking steps to inactivate prions is always a work in progress as new therapeutic products come into production and new methods to inactivate prions are discovered. The tried-and-true laboratory method of prion sterilization (1-hour exposures to either undiluted bleach or 1 N sodium hydroxide followed by steam autoclaving at 3 atmospheres pressure for 20 minutes) is applicable only to nonfragile instruments and not at all to living tissues. The surprising resistance of dura mater to 0.1 N sodium hydroxide (*17*) and of growth hormone to 6 M urea (*18*) led to their incorporation into processing protocols before being replaced by nondural tissue or synthetic patches and recombinant hormone. To reduce infectivity, blood, blood products, and other fluids can be subjected to nanofiltration and prion-affinity ligands (*19*–*22*), which should also be applicable to other biological products, for example, vaccine and stem cell cultures, should they be susceptible to infection (*23*). Fragile instruments such as endoscopes and electrodes remain a challenge, but new and gentler methods— alkaline cleaning solutions, phenolics, and gaseous hydrogen peroxide—have proven harmless to instruments and give a high, if not always complete, degree of prion inactivation (*24*–*26*).

The ongoing refinement of a quaking-induced conversion detection of the misfolded prion protein holds the best prospect of evolving into a sensitive and practical tool, but it has yet to be validated in blind testing of plasma from symptomatic patients or in presymptomatic persons, even more rigorous but necessary (*27*,*28*). It may be necessary to use scrapie-infected animals for presymptomatic validation because only 1 group of humans could furnish appropriate samples—asymptomatic carriers of CJD-inducing mutations—and putting together and testing a reasonable number of such samples will take years to accomplish.

The total numbers of cases for the 2 major causes of iatrogenic CJD during the past 40 years (226 growth hormone cases and 228 dura mater cases) are amazingly close and are likely to remain so after the few additional long-incubating cases finally surface in the next few years. The combination of appropriate blood donor deferrals and the incorporation of tissue, fluid, and instrument infectivity–reduction steps should continue to hold the sources of potential iatrogenic disease to a minimum until such time as a practical screening test for inapparent infection is validated for human use.

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Dr Brown spent his career at the National Institutes of Health in the Laboratory of Central Nervous System Studies conducting research on the transmissible spongiform encephalopathies, especially with respect to epidemiology, iatrogenic CJD, disinfection, and blood infectivity. He currently chairs a scientific advisory committee for the Laboratoire Français du Fractionnement et des Biotechnologies in Les Ulis, France, and advises the Centre à l'Energie Atomique in Fontenay-aux-Roses, France.

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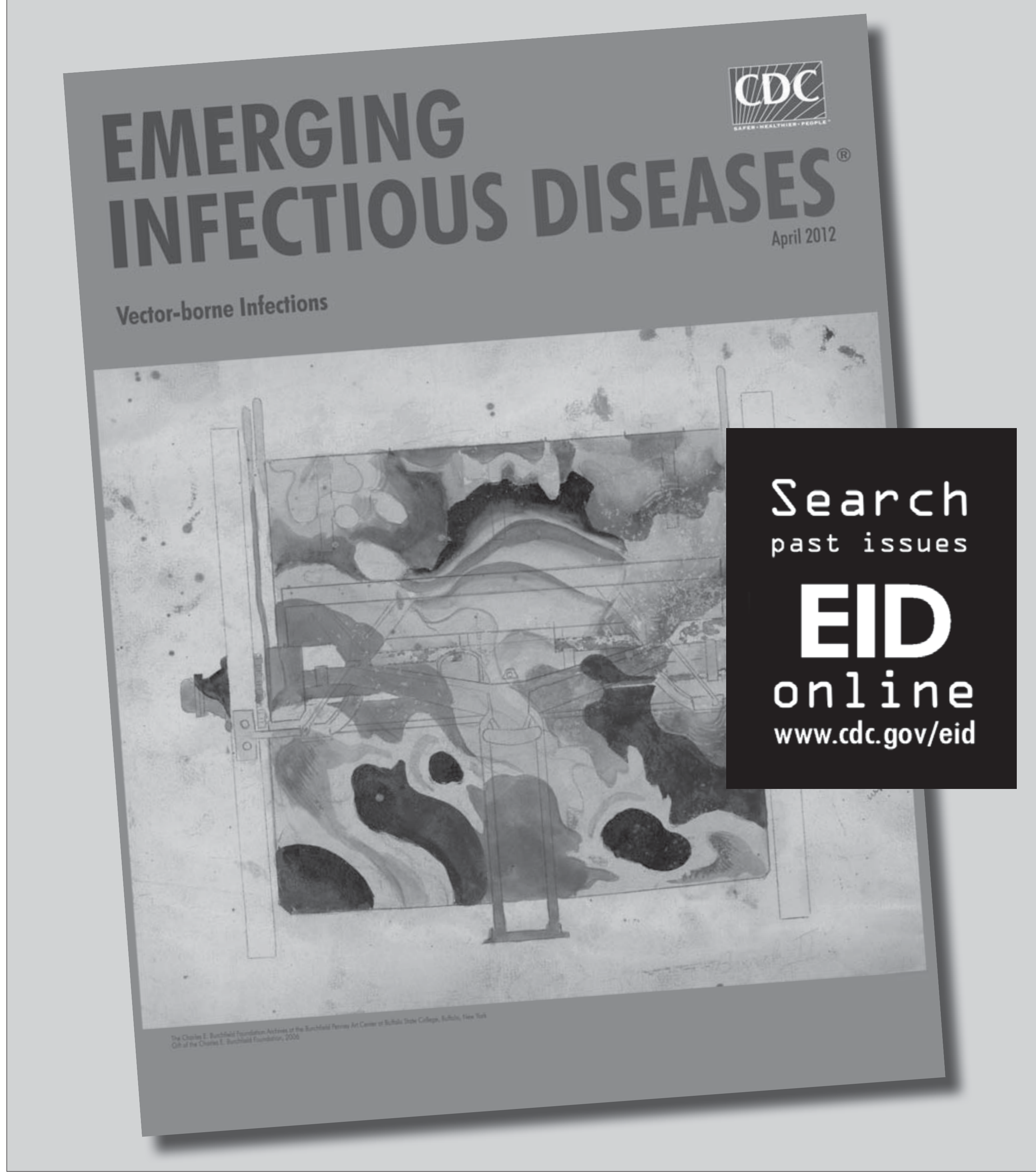

# Pretransplant Fecal Carriage of Extended-Spectrum β-Lactamase–producing *Enterobacteriaceae* and Infection after Liver Transplant, France

Frédéric Bert, Béatrice Larroque, Catherine Paugam-Burtz, Federica Dondero, François Durand, Estelle Marcon, Jacques Belghiti, Richard Moreau, and Marie-Hélène Nicolas-Chanoine

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**Learning Objectives**

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

- Describe incidence and characteristics of ESBLE infections following liver transplantation, based on a 10-year study
- Describe clinical outcomes of ESBLE infections following liver transplantation, based on a 10-year study
- Describe the effect of ESBLE fecal carriage on risk for ESBLE infections following liver transplantation, and other risk factors for ESBLE infections following liver transplantation.

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Extended-spectrum β-lactamase–producing *Enterobacteriaceae* isolates (ESBLE) are emerging pathogens that confer resistance to antimicrobial drugs. We conducted a 10-year study in France (January 2001–April 2010) to investigate the incidence of and risk factors for ESBLE infections after liver transplant. Of 710 transplant patients screened preoperatively for ESBLE fecal carriage, 5.5% had ESBLE infection develop within 4 months after surgery; patients with pretransplant ESBLE fecal carriage were more likely to have infection develop than were noncarriers. Typing showed extensive genetic diversity, with a large predominance of CTX-M enzymes. Independent predictors of ESBLE infection were pretransplant fecal carriage, Model for End Stage Liver Disease score ≥25, and return to surgery. Our results indicate that the influx of preoperatively acquired ESBLE isolates into the hospital outweighs cross-transmission in the epidemiology of ESBLE infections after liver transplant. Transplant candidates should be systematically screened for carriage, and posttransplant infection in carriers should be treated with carbapenems.

Extended-spectrum β-lactamase (ESBL)–producing *Enterobacteriaceae* (ESBLE) isolates, notably ESBL-producing *Escherichia coli* isolates, have emerged worldwide as a frequent cause of infection in hospitals and in the community (*1*,*2*). ESBLs confer resistance to all β-lactam agents except carbapenems and cephamycins and are frequently associated with resistance to other classes of antimicrobial drugs, including aminoglycosides and fluoroquinolones (*2*,*3*). Therapeutic options for infections caused by these multidrug-resistant organisms are therefore limited, which may result in delayed effective therapy and increased risk for death (*4*,*5*). Identifying risk factors for ESBLE infections enables identification of patients who should receive empirical treatment targeted to these organisms.

ESBLE infections may pose a particularly serious threat in profoundly immunosuppressed patients, such as transplant recipients, who are often empirically treated for postoperative infections. Solid organ transplant has been shown to be a risk factor for nosocomial ESBLE bacteremia (*6*), but little research has been conducted to identify risk factors for ESBLE infections among these patients, and previous studies included only kidney transplant recipients (*7*,*8*). Liver transplant (LT) recipients are particularly at risk for bacterial infections because of the severity of illness associated with preoperative end-stage liver disease and the technical complexity of surgery (*9*). After LT, bacterial infections occur in 35%–70% of patients; these infections are predominantly caused by *Enterobacteriaceae* (*10*–*13*). Although recent studies from Spain and China have reported alarmingly high rates of ESBL production in isolates from LT recipients (*14*–*16*), to our knowledge, the epidemiology of ESBLE infections among these high-risk patients in a nonepidemic setting has not been investigated.

We assessed the incidence of, risk factors for, and molecular epidemiology of ESBLE infections in LT recipients. More specifically, we investigated whether ESBLE pretransplant fecal carriage was an independent predictor of ESBLE infection after LT.

## Materials and Methods

### Study Population

An observational study of patients undergoing LT at Beaujon Hospital (Clichy, France) was conducted from January 1, 2001, through April 30, 2010. During this period, 734 patients underwent LT, and pretransplant screening for ESBLE fecal carriage was routinely performed as part of an infection control program. Rectal swab specimens were obtained preoperatively on the day of surgery from 710 of the 734 LT recipients; these 710 patients constituted the study population (Table 1).

During the study period, standard antimicrobial drug prophylaxis consisted of cefoxitin administered intraoperatively as described (*17*); primary immunosuppressive therapy consisted of tacrolimus, corticosteroids, and mycophenolate mofetil (*17*). Throughout the study period, isolation procedures were applied to patients infected or colonized with ESBLE isolates until they were discharged from the hospital. More specifically, these patients were placed in individual rooms, and contact isolation precautions, including the use of gowns and gloves, were implemented.

### Definition and Data Collection

ESBLE infections occurring within 4 months after LT were investigated. Infections were defined on the basis of clinical criteria and the isolation of an ESBLE isolate from a clinically significant site. Bacteremia was defined as a positive peripheral blood culture bottle result (*13*,*18*). Intraabdominal infection was considered to be present when an ESBLE isolate was cultured from biliary fluid, from peritoneal fluid containing ≥250 polymorphonuclear cells/mm$^3$, or from an intraabdominal collection drained surgically or by percutaneous aspiration (*19*). Pneumonia was diagnosed on the basis of radiographic findings, clinical signs, and culture of ≥10$^3$/mL of an ESBLE isolate from a protected distal aspirate (*20*). Urinary tract infection was diagnosed on the basis of urine leukocyte count and a positive culture ≥10$^4$ cells/mL (*21*).

Data were collected in a prospectively maintained database. Preoperative data were age, gender, underlying liver disease, HIV infection, Model for End Stage Liver Disease (MELD) score, pretransplant ESBLE fecal carriage, stay in intensive care unit ≥48 h before surgery, hospitalization in the previous 6 months, and any history of

Table 1. Characteristics of 710 liver transplant recipients included in study of ESBLE pretransplant fecal carriage and posttransplant infection, France, January 2001–April 2010*

| Characteristic | Value |
|---|---|
| Age, y, mean ± SD | 50 ± 11 |
| Male sex | 490 (69) |
| Viral hepatitis | 261 (36.7) |
| Hepatocellular carcinoma | 221 (31.1) |
| Alcoholism | 196 (27.6) |
| Acute liver failure | 74 (10.4) |
| Biliary disease | 61 (8.6) |
| HIV infection | 21 (3) |
| MELD score, mean ± SD | 18 ± 9 |

*Values are no. (%) patients except as indicated. ESBLE, extended spectrum β-lactamase–producing *Enterobacteriaceae*; MELD, Model for End-stage Liver Disease.

bacteremia, spontaneous bacterial peritonitis, or both in the previous 6 months. Intraoperative variables were duration of surgery, cold ischemia time, combined kidney and liver transplant, type of donor (deceased or living), number of packed red blood cell units transfused, and type of biliary reconstruction (duct-to-duct or Roux-en-Y anastomosis). Postoperative data were retransplantation, return to surgery, and acute renal failure.

Outcome was assessed by death within 28 days after transplant, death while hospitalized, and death related to infection. Death was considered be related to infection when ESBL infection was the immediate cause of death or when it initiated a sequence of events leading to multiorgan failure and death.

### Microbiological Studies

A rectal swab specimen was routinely obtained before LT and plated onto a selective medium consisting of Drigalsky agar plates with 1 µg/mL cefotaxime during 2001–2004 and commercialized agar plates (bioMérieux, Marcy l'Etoile, France) during 2004–2010. Plates were incubated for 24–48 h. A single sample of each distinct type of colony growing on the selective media was picked and further studied. Isolates were identified to the species level by API 20E strips (bioMérieux), and ESBL production was confirmed by double disk synergy test (*22*). This test was performed by placing disks of cefotaxime, ceftazidime, cefepime and aztreonam on Mueller-Hinton agar at a distance of 30 mm, center to center, from an amoxicillin/clavulanate disk. In case of ambiguous results for ampicillin C producers, the presence of an ESBL was further investigated by a double disk synergy test with a disk spacing of 20 mm and another test performed on Mueller-Hinton agar containing cloxacillin (*22*).

*Enterobacteriaceae* isolates from clinical specimens were also identified by the API 20E strips, and antimicrobial drug susceptibility was tested by the Mueller-Hinton agar diffusion method and interpreted according to the recommendations of the Committee of the French Society for Microbiology (*23*). Clinical isolates were screened for ESBL production by the double-disk synergy test as described for rectal isolates. Rectal and clinical isolates from were kept frozen at −70°C until subcultured for strain and ESBL typing.

### Strain Typing

The clonal relatedness of ESBLE clinical isolates was studied by the enterobacterial repetitive intergenic consensus sequence type 2 (ERIC-2) PCR fingerprinting method (*24*). Electrophoretic banding patterns were compared by visual inspection. For patients who had ESBLE preoperative fecal carriage and subsequently had infection develop, the isolate from the rectal swab specimen and the isolate from the infected site were compared by using ERIC-2 PCR.

### β-Lactamase Typing

The main types of ESBL are SHV-, TEM-, and CTX-M ESBLs. These types were identified by using the Check-KPC ESBL microarray system (Check-Points, Wageningen, the Netherlands), according to the manufacturer's instructions (*25*,*26*).

### Statistical Analysis

The prevalence of pretransplant ESBLE fecal carriage and subsequent ESBLE infections was compared among 4 periods (2001–2003, 2004–2006, 2007–2008, and 2009–2010) by a test for trend. Univariate analysis was used to identify associations between each of the preoperative, intraoperative, and postoperative variables and the occurrence of ESBLE infections. The $\chi^2$ test and, for small numbers, the Fisher exact test were used for comparison of categorical data. Continuous data were compared by Student *t* test; $p \leq 0.05$ was considered significant. We performed stepwise logistic regression analysis to identify significant correlates of ESBLE infections: variables with a p value <0.1 were entered into the model. Potential interactions between covariates were considered. Data analyses were performed with SAS version 9.2 (SAS Institute, Inc., Cary, NC, USA).

## Results

### Preoperative ESBLE Fecal Carriage

Of the 710 patients screened preoperatively, 29 (4.1%) had ESBLE pretransplant fecal carriage; 4 patients each had 2 distinct ESBLE isolates. Species distribution of the 33 fecal isolates was as follows: *E. coli* (n = 21), *Enterobacter cloacae* (n = 5), *Klebsiella pneumoniae* (n = 4), *K. oxytoca* (n = 1), *Citrobacter koseri* (n = 1), and *C. freundii* (n = 1). The rate of pretransplant ESBLE fecal carriage increased significantly, from 0% to 10.6%, during the study period (Table 2).

Table 2. Temporal evolution of ESBLE pretransplant fecal carriage and posttransplant infection, France, by time period, January 2001–April 2010*

| Variable | No. (%) patients | | | | p value† |
|---|---|---|---|---|---|
| | 2001–2003, n = 185 | 2004–2006, n = 196 | 2007–2008, n = 188 | 2009–2010, n = 141 | |
| Pretransplant ESBLE fecal carriage | 0 | 5 (2.5) | 9 (4.8) | 15 (10.6) | <0.0001 |
| Posttransplant ESBLE infection | 3 (1.6) | 9 (4.6) | 9 (4.8) | 18 (12.8) | <0.0001 |

*ESBLE, extended spectrum β-lactamase–producing *Enterobacteriaceae*.
†Comparison among the 4 periods by test of trend.

### ESBLE Infections

Of the 710 patients included in the study, 39 (5.5%) had ESBLE infection develop within 4 months after LT. The incidence rate of a first episode of ESBL infection was 5.1 cases/10,000 posttransplant days. The rate of infected patients increased significantly during the study period, from 1.6% during 2001–2003 to 12.8% during 2009–2010 (Table 2).

The median time from LT to ESBLE infection was 15 days (range 3–105 days). ESBLE infection was the first posttransplant infection in 15/39 patients. The sites of ESBL infection were as follows: intraabdominal (n = 19), urinary tract (n = 15), respiratory tract (n = 6), bloodstream (n = 2), and skin and soft tissues (n = 1). Eight patients had secondary bacteremia: 3 patients with peritonitis, 3 patients with cholangitis, 1 with pneumonia, and 1 with cellulitis. Overall, 10 (25.6%) of the 39 infected patients were bacteremic.

The predominant causative agent of infection in these patients was *E. coli* (n = 16), followed by *E. cloacae* (n = 11), *K. pneumoniae* (n = 5), *K. oxytoca* (n = 3), *C. koseri* (n = 2), and *Enterobacter aerogenes* (n = 2). The susceptibility rates of the 39 isolates are shown in Table 3. All isolates were susceptible to imipenem.

Six patients died within 28 days after ESBLE infection developed (28-day mortality rate 15.4%); 11 patients died during hospitalization (in-hospital mortality rate 28.2%). Death was related to the infection in 8 patients (infection-related mortality rate 20.5%). The in-hospital mortality rate was significantly higher in patients with ESBLE infection than in those without ESBLE infection (28.2% vs. 15.9%; p = 0.0455).

### Association between Fecal Carriage and Infection

ESBLE infection occurred in 13 (44.8%) of the 29 patients with pretransplant fecal carriage versus 26 (3.8%) of the 681 noncarriers (p<0.0001). Ten (84.6%) of the 13 patients with fecal carriage had ESBL infection develop within 14 days after LT. The median time to onset of infection was significantly shorter among patients with pretransplant fecal carriage than among those without carriage (9 days vs. 25 days; p = 0.0007). For the 13 carriers in whom ESBLE infection developed, the infecting isolate belonged to the same species as the fecal (or 1 of the fecal) isolate(s). In 10 of these 13 pairs of isolates, ERIC-2 PCR typing showed that the fecal isolate and the infecting isolate had identical patterns. An example of banding patterns is shown in the Figure.

### Genetic Relatedness and Types of ESBLs

Overall, 31 ERIC-2 PCR profiles were observed among the 39 ESBLE clinical isolates (Table 3). Six of these patterns were observed in 2 isolates each (2 *E. coli*, 2 *K. pneumoniae*, 1 *E. cloacae*, 1 *C. koseri*), and 1 pattern was shared by the 3 *K. oxytoca* isolates. The remaining 24 patterns were observed in a single isolate each.

The Check-KPC ESBL microarray analysis (Check-Points) was used to identify the type(s) of ESBL in 37/39 tested isolates (Table 3). Nine of the isolates produced different types of ESBL, 8 isolates with 2 types and 1 isolate with 3 types; a single type of ESBL was detected in the remaining 28 isolates. Members of the CTX-M family, in particular those of the CTX-M-1 group, were most frequently detected (Table 3). Overall, enzymes of the CTX-M family were observed in 31 (79.5%) of the 39 isolates tested, including all *E. coli* isolates. The type of ESBL could not be identified in 2 isolates; this result may have resulted from plasmid loss, because the double-disk synergy test result was negative after subculture.

### Risk Factor Analysis

The characteristics of patients with and without ESBL infection are compared in Table 4. Variables significantly associated with infection in the univariate analysis were pretransplant ESBLE fecal carriage, acute liver failure, increased MELD score, prolonged hospitalization during the 6 months before LT, preoperative intensive care stay ≥48 h, return to surgery, and postoperative acute renal failure. The 10 variables with a p value <0.1 were entered in the stepwise logistic regression. In the final model, pretransplant ESBLE fecal carriage (odds ratio [OR] 18.4), MELD score ≥25 (OR 2.9), and return to surgery (OR 2.7) were independent predictors of ESBLE infection (Table 5). No significant interactions between the covariates kept in the model were found.

## Discussion

We demonstrated a progressive and significant increase in the incidence of pretransplant ESBLE fecal carriage and posttransplant infection among LT patients in our

transplant center over the past decade. We also identified pretransplant fecal carriage as an independent risk factor for subsequent ESBLE infection. Lastly, we found high clonal diversity and a vast majority of CTX-M-1 group and CTX-M-9 group enzymes among our isolates.

Recent studies suggest that nonhospitalized patients are increasingly important reservoirs of ESBLE isolates; these community reservoirs may play a role in the epidemiology of ESBLE infections in hospital settings (*27–30*). Harris et al. reported that 2% of patients admitted to intensive care were colonized by an ESBL-producing *E. coli* or *Klebsiella* spp. isolate (*31*). In 2 other recent studies, the rates of ESBLE fecal carriage at hospital admission were 10.8% and 8%, respectively (*29*,*32*). Overall, the rate of pretransplant carriage was 4.1% in our liver transplant recipients, and a significant increase, from 0% to 10.6%, was noted over the study period. These isolates may have been acquired in the community or during previous hospitalizations.

Overall, 5.5% of patients in our study had ESBLE infection develop within 4 months after LT. This infection rate increased in similar proportions to the pretransplant colonization rate over time, reaching 12.8% during 2009–2010. We found a significant association between fecal colonization status and occurrence of subsequent infection. Posttransplant ESBLE infection developed in ≈45% of carriers; most infections occurred within 14 days after LT, and molecular typing showed that the infecting isolate was identical to the isolate from the prettransplant fecal swab for most of these patients. This finding is consistent with previous studies in nontransplant patients that found prior

Table 3. Antimicrobial drug susceptibility, ERIC-2 PCR type, and β-lactamase gene content of the 39 clinical isolates of ESBL-producing *Enterobacteriaceae* identified, France, January 2001–April 2010*

| Species | Year of isolation | Susceptibility | | | | ERIC-2 PCR type | β-lactamase types† | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | TZP | FOX | AMK | CIP | | CTX-M-1 group | CTX-M-9 group | ESBL TEM | Non-ESBL TEM | ESBL SHV | Non-ESBL SHV |
| *E. coli* | 2003 | S | S | S | R | 1 | + | – | – | + | – | – |
| | 2004 | R | S | S | R | 2 | + | – | – | + | – | – |
| | 2004 | R | R | S | S | 3 | + | – | – | – | – | – |
| | 2007 | R | R | R | R | 4 | + | – | – | – | – | – |
| | 2007 | S | S | S | R | 5 | + | – | – | + | – | + |
| | 2008 | S | S | R | R | 6 | + | – | – | – | – | – |
| | 2008 | R | S | S | R | 7 | + | – | – | – | – | – |
| | 2008 | S | S | S | S | 8 | + | – | – | + | – | + |
| | 2009 | S | S | R | R | 9 | + | – | – | + | – | – |
| | 2009 | S | S | S | R | 10 | – | + | – | – | – | – |
| | 2009 | S | R | R | R | 9 | + | – | – | + | – | – |
| | 2009 | R | S | R | R | 11 | + | + | – | + | – | – |
| | 2009 | R | S | S | R | 4 | + | – | – | + | – | – |
| | 2010 | R | S | R | R | 12 | + | – | – | – | – | – |
| | 2010 | S | S | S | S | 13 | + | – | – | – | – | – |
| | 2010 | R | R | R | R | 14 | + | – | – | – | – | – |
| *K. pneumoniae* | 2008 | R | S | S | R | 15 | + | – | – | – | – | + |
| | 2008 | R | S | S | R | 16 | + | – | – | – | – | + |
| | 2010 | R | R | R | R | 17 | + | – | – | + | – | + |
| | 2010 | R | R | R | R | 17 | + | – | – | + | – | + |
| | 2010 | R | S | S | R | 15 | + | – | – | – | + | – |
| *K. oxytoca* | 2009 | R | R | R | R | 18 | – | – | – | – | + | – |
| | 2009 | R | R | R | R | 18 | – | – | – | – | + | – |
| | 2009 | R | R | R | R | 18 | + | – | – | + | – | – |
| *C. koserii* | 2006 | S | R | R | R | 19 | – | + | + | – | – | – |
| | 2006 | S | R | R | R | 19 | – | + | + | – | – | – |
| *E. cloacae* | 2002 | R | R | R | R | 20 | – | – | – | + | - | – |
| | 2004 | R | R | R | R | 21 | + | – | – | + | + | – |
| | 2004 | R | R | S | R | 22 | – | – | – | – | + | – |
| | 2004 | R | R | I | R | 23 | – | – | – | – | + | – |
| | 2005 | R | R | R | R | 24 | – | – | – | + | – | – |
| | 2008 | S | R | R | R | 25 | + | – | – | + | + | – |
| | 2008 | S | R | R | R | 21 | – | – | – | + | + | – |
| | 2009 | R | R | S | R | 26 | + | – | – | + | – | – |
| | 2009 | R | R | S | R | 27 | + | – | – | – | – | – |
| | 2009 | S | R | R | R | 28 | + | – | – | – | + | – |
| | 2010 | S | R | R | R | 29 | – | – | – | + | + | – |
| *E. aerogenes* | 2002 | R | R | R | R | 30 | + | – | + | – | – | – |
| | 2005 | R | R | R | R | 31 | + | + | – | + | + | – |

*ERIC-2, enterobacterial repetitive intergenic consensus sequence type 2; ESBL, extended-spectrum β-lactamase; TZP, piperacillin/tazobactam; FOX, cefoxitin; AMK, amikacin; CIP, ciprofloxacin; *E. coli*, *Escherichia coli*; S, susceptible; R, resistant; *K. pneumoniae*, *Klebsiella pneumoniae*; *K. oxytoca*, *Klebsiella oxytoca*; *C. koserii*, *Citrobacter koserii*; *E. cloacae*, *Enterobacter cloacae*; *E. aerogenes*, *Enterobacter aerogenes*.
†The types of β-lactamase were identified by the Check-KPC ESBL microarray system (Check-Points, Wageningen, the Netherlands).

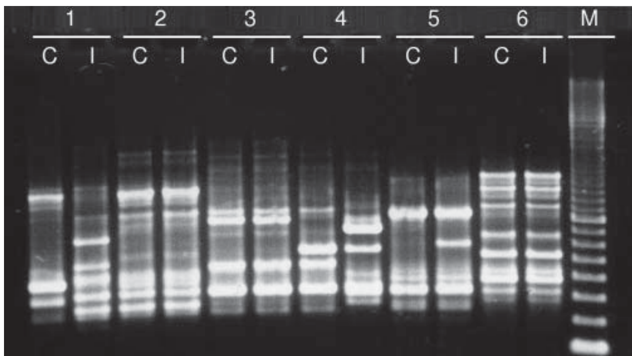


Figure. Enterobacterial repetitive intergenic consensus sequence type 2 PCR patterns of pairs of *Escherichia coli* isolates from 6 patients examined during study of extended-spectrum β-lactamase–producing *Enterobacteriaceae* infection among liver transplant recipients, France, January 2001–April 2010. The pretransplant colonizing isolate (C) and the posttransplant infecting isolate (I) show identical patterns for patients 2, 3, and 6 and different patterns for patients 1, 4, and 5. M, molecular mass standard.

colonization with an ESBLE isolate confers an increased risk for hospital-acquired infection with the same organism (*29*,*33*,*34*). In contrast, Arnan et al. recently found that fecal carriage of ESBL-producing *E. coli* was not associated with infection in neutropenic patients who had hematologic malignancies (*35*).

Differences in infection rates on the basis of pretransplant colonization status may indicate that fecal carriage plays a direct role in the pathogenesis of ESBLE infection or may only reflect differences in preoperative host characteristics. In this study, we used a multivariate logistic regression analysis to control for the effects of confounding variables such as the severity of underlying liver disease. The results indicate that pretransplant fecal carriage is an independent risk factor for ESBLE infection after LT. Other predictors of infection in the multivariate analysis were high preoperative MELD score and return to surgery, 2 factors previously identified as risk factors for *Enterobacteriaceae* bacteremia in LT recipients (*18*).

Molecular typing of our isolates showed extensive genetic diversity. This finding strongly suggests that in-hospital spread of specific clones was not the cause of the increase in the frequency of ESBLE infections in our transplant center. An active infection-control program for multidrug-resistant organisms was in place during the study period, including isolation procedures for preventing patient-to-patient transmission of ESBLE isolates. Similarly, Harris et al. reported that cross-transmission was not an important cause of acquisition of ESBL-producing *E. coli* in the intensive care unit setting (*36*). However, it is noteworthy that ESBLE infection occurred in 26 patients whose pretransplant fecal carriage screening tests were negative, which suggests postoperative acquisition. An alternative explanation is that rectal screening may have failed to detect colonization in patients with a low intestinal inoculum.

Table 4. Comparison of patients with and without ESBLE infection, by univariate analysis, France, January 2001–April 2010*

| Variable | Patients with ESBLE infection, n = 39 | Patients without ESBLE infection, n = 671 | p value |
|---|---|---|---|
| Preoperative data | | | |
| Mean age, y | 49 | 50 | 0.55 |
| Male sex | 24 (61.5) | 466 (69.4) | 0.30 |
| ESBLE fecal carriage | 13 (33.3) | 16 (2.4) | <0.0001 |
| Viral hepatitis | 13 (33.3) | 248 (37.1) | 0.64 |
| Hepatocellular carcinoma | 7 (17.9) | 214 (32.3) | 0.061 |
| Alcoholism | 16 (41) | 219 (32.6) | 0.28 |
| Acute liver failure | 11 (28.2) | 63 (9.4) | 0.0011 |
| HIV infection | 1 (2.5) | 20 (3) | 1 |
| MELD score ≥25 | 20 (51.3) | 143 (21.4) | <0.0001 |
| Intensive care stay ≥48 h† | 14 (35.9) | 80 (11.2) | <0.0001 |
| Hospital stay ≥10 d† | 20 (51.3) | 185 (27.6) | 0.0015 |
| Spontaneous bacterial peritonitis† | 7 (17.9) | 63 (9.4) | 0.082 |
| Bacteremia† | 5 (12.8) | 70 (10.4) | 0.59 |
| Intraoperative data | | | |
| Mean duration of surgery, min | 488 | 509 | 0.12 |
| Mean cold ischemia time, min | 472 | 484 | 0.47 |
| Kidney transplant | 2 (5.1) | 39 (5.8) | 1 |
| Living donor | 3 (7.9) | 56 (8.4) | 1 |
| Mean no. units red blood cells transfused | 5.9 | 4.5 | 0.11 |
| Roux-en-Y anastomosis | 4 (10.2) | 46 (7.6) | 0.53 |
| Postoperative data | | | |
| Retransplantation | 3 (7.7) | 40 (6) | 0.72 |
| Return to surgery | 23 (59) | 228 (34) | 0.0015 |
| Acute renal failure | 24 (66.7) | 238 (38.1) | 0.0007 |

*Values are no. (%) except as indicated. ESBLE, extended-spectrum β-lactamase–producing *Enterobacteriaceae*; MELD, Model for End-stage Liver Disease.
†In the 6 mo before liver transplant.

Table 5. Risk factors for ESBLE infection after liver transplant, by stepwise logistic regression analysis, France, January 2001–April 2010*

| Variable† | OR (95% CI) | p value |
|---|---|---|
| Pretransplant ESBLE fecal carriage | 18.4 (7.1–47.5) | <0.0001 |
| MELD score ≥25 | 2.9 (1.4–6.2) | 0.0053 |
| Return to surgery | 2.7 (1.3–5.9) | 0.011 |

*ESBLE, extended-spectrum β-lactamase-producing *Enterobacteriaceae*; OR, odds ratio; MELD, Model for End-stage Liver Disease.
†Other factors initially in the model were hepatocellular carcinoma, acute liver failure, pretransplant ICU stay ≥48h, hospital stay ≥10 d in the 6 mo before transplant, spontaneous bacterial peritonitis in the 6 mo before transplant, red blood cells transfused, and posttransplant acute renal failure.

Prior exposure to antimicrobial drugs is a well-defined risk factor for ESBLE colonization and infection in hospitalized patients and in the community (*6*,*29*–*33*,*35*,*37*). Patients undergoing LT are particularly likely to be exposed to antimicrobial drugs, but this exposure is difficult to evaluate because all patients receive intraoperative prophylaxis, and many receive antimicrobial drugs in the early postoperative period for suspected or documented bacterial infections. In this study, ESBLE infection was not the first postoperative infection in 24/39 infected patients, which indicates previous antimicrobial drug use after LT. Moreover, antimicrobial drugs given for pretransplant infections, which occur in 30%–35% of candidates for LT (*38*), may have predisposed them to ESBLE colonization and subsequent infection. Pretransplant antimicrobial drug exposure also includes norfloxacin prophylaxis in patients with a history of spontaneous bacterial peritonitis. Unfortunately, we did not have access to information about all antimicrobial drugs that patients may have received as outpatients or during hospitalizations in other institutions during the waiting period. Further studies are therefore needed to identify predictors of pretransplant ESBLE fecal carriage among LT recipients.

Our results have implications for the treatment of infections in LT recipients. Because ESBLE infections may be associated with inappropriate initial therapy and increased risk for death, the identification of high-risk patients is useful when choosing empiric antimicrobial drugs treatment. Therefore, we suggest that candidates for LT be systematically screened for ESBLE fecal carriage. Carbapenems should be included in initial empiric therapy for infections in LT recipients who have documented ESBLE fecal carriage.

The main limitation of our work is that it was a single-center study, which means the results may not be applicable to settings with a different epidemiologic context. However, our center is one of the largest LT centers in France and receives referrals from throughout the country. Moreover, our findings are consistent with most recent reports on the epidemiology of ESBLE infections worldwide. During the past decade, *E. coli* has become the most prevalent species among ESBLE isolates, and CTX-M enzymes have replaced TEM and SHV enzymes as the most common types of ESBLs (*1*,*2*). Similarly, *E. coli* was the most frequent species identified among our isolates, and ESBL typing revealed a large predominance of CTX-M-1 group enzymes. Therefore, we assume our findings can be generalized to other LT centers that have similar infection control strategies. Another limitation of our study is that some ESBLE isolates may have been unidentified in patients carrying different strains of ESBL-producing *E. coli*.

In summary, preoperative fecal carriage is an independent predictor of ESBLE infection after LT. The influx of preoperatively acquired ESBLE isolates into the hospital outweighs posttransplant cross-transmission in the epidemiology of these infections. Given the worldwide ongoing diffusion of ESBLE isolates outside hospitals, the proportion of LT recipients in whom ESBLE infections develop is likely to further increase over time, in spite of adequate infection control measures. Therefore, these multidrug-resistant organisms may become a major threat in LT centers in the near future.

Dr Bert is a clinical microbiologist at Beaujon Hospital. His research interests include infections in patients with cirrhosis and in liver transplant recipients.

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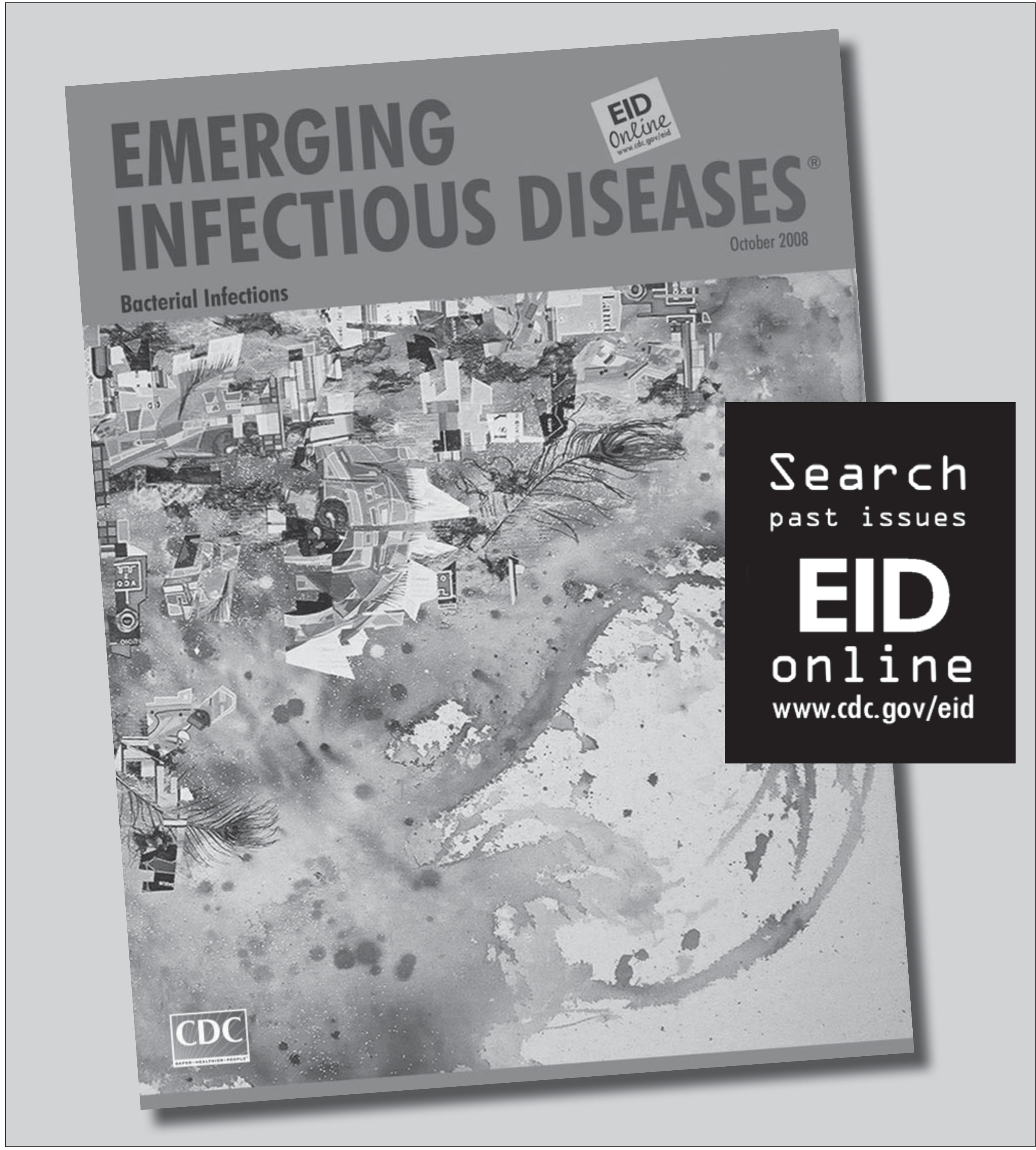

# Trends in Invasive Infection with Methicillin-Resistant *Staphylococcus aureus*, Connecticut, USA, 2001–2010

**James L. Hadler, Susan Petit, Mona Mandour, and Matthew L. Cartter**

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**Learning Objectives**

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

- Identify the most common classification of MRSA recorded in 2001 to 2010
- Evaluate the epidemiology of MRSA between 2001 and 2010
- Distinguish the classification of MRSA which increased between 2001 and 2010
- Analyze the bacteriology of MRSA infections in the current study

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We examined trends in incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) infections in Connecticut, with emphasis on 2007–2010, after legislation required reporting of hospital infections. A case was defined as isolation of MRSA from normally sterile body sites, classified after medical record review as hospital onset (HO), community onset, health care–associated community onset (HACO), or community-associated (CA). Blood isolates collected during 2005–2010 were typed and categorized as community- or health care–related strains. During 2001–2010, a total of 8,758 cases were reported (58% HACO, 31% HO, and 11% CA), and MRSA incidence decreased ($p<0.05$) for HACO and HO, but increased for CA. Significant 3- to 4-year period trends were decreases in all MRSA (–18.8%), HACO (–12.8%), HO (–33.2%), and CA (–12.7%) infections during 2007–2010, and an increase in CA infections during 2004–2006. Decreases in health care–related isolates accounted for all reductions. Hospital infections reporting may have catalyzed the decreases.

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Methicillin-resistant *Staphylococcus aureus* (MRSA) was first identified in 1961 in England (*1*). The pathogen became a growing concern in US hospitals as *S. aureus* infections steadily increased, from causing 2.4% of nosocomial infections in 1975 to 29% by 1991 (*2*). In the mid-1990s, reports of community-onset infections caused by MRSA increased (*3*,*4*), including in children with no health care–associated risk factors (*5*,*6*). A 1998 population-based study in Connecticut showed that MRSA accounted for 23% of all blood isolates from persons with community-onset *S. aureus* infection admitted to the hospital (*7*). Subsequent studies showed that 2 epidemiologically distinct, although overlapping, MRSA problems were occurring, one in the hospital associated with highly antimicrobial drug–resistant MRSA strains and the other in the community. The infections in the community were fueled by 2 factors: persons' exposure during health care to hospital-generated strains and the emergence and transmission of strains in the community that are less resistant to antimicrobial drugs (*8*,*9*).

In the late 1990s, the Emerging Infections Program (EIP), funded by the Centers for Disease Control and Prevention (CDC), became interested in better defining MRSA dynamics from a population-based perspective and established pilot surveillance systems at sites in 4 states for either all MRSA infections (Minnesota, Georgia, Maryland) or for invasive disease (Connecticut) (*8*,*10*,*11*). On the basis of findings from these efforts, formal population-based surveillance for invasive MRSA began in mid-2004 in 9 EIP sites comprising 16.5 million persons to obtain a national picture of the magnitude and trends in the most serious MRSA infections. A summary of data from 2005, the first full year of EIP invasive disease surveillance, was published in 2007 and revealed the full magnitude of invasive MRSA in the United States: ≈94,360 persons had invasive infections in 2005, and 18,650 patients died while hospitalized (*12*). This study also demonstrated that most MRSA infections (85%) were health care–associated, with 69% occurring in the community rather than in the hospital.

Since 2004, health care–associated infections have received increasing national attention. Efforts by patients' advocate groups beginning in 2004 have resulted in the passage of legislation mandating that hospitals report infections to their state health department in 27 states, and in at least 12 states, legislation related to MRSA reporting, screening, or producing MRSA infection control plans (*13*). Since then, state-level involvement in health care–associated infections, including MRSA, has become commonplace. Given that hospital and community factors can affect trends in MRSA incidence, determining their net population level (as well as hospital-level) effects is crucial.

Connecticut began population-based surveillance for invasive MRSA infection in 2001, thus providing an opportunity to examine trends over a 10-year period. Our objectives in this analysis were twofold: 1) to describe the epidemiology of invasive MRSA in Connecticut and trends over time by place of illness onset (community vs. hospital) and relationship to health care, and 2) to describe MRSA strain subtypes associated with place of onset and trends over time.

## Methods

### Study Design

Connecticut participates in the Active Bacterial Core Surveillance project of the EIP at CDC. As part of this surveillance project, statewide active surveillance for invasive MRSA began in 2001. A case of invasive MRSA infection was defined as isolation of MRSA from a normally sterile body site (per Active Bacterial Core Surveillance protocol) (*10*) of a Connecticut resident at a clinical laboratory in Connecticut. Cases were identified through mandated statewide reporting by laboratories and clinicians. To verify completeness of reporting, we routinely audited all in-state hospital and reference laboratories. Cases for which a diagnosis was made out of state and those with cultures considered to be contaminants on the basis of a medical record discharge summary were excluded. Recurrent MRSA infections (positive culture taken >30 days after the initial positive culture in the same patient) were also excluded, both because the focus of this analysis was to ascertain the number and incidence of unique persons affected and because tracking for recurrence only began in 2004.

Medical chart reviews were conducted for hospitalized patients by using a standardized case report form. Data collected included demographics, infection type, underlying illnesses, and risk factors for infection as described below.

### Variable Definitions

Cases were classified into 3 mutually exclusive categories according to place of onset and relationship to health care. Hospital-onset (HO) MRSA cases were those for which cultures were collected >2 days after hospital admission (day of admission = day 0). Health care–associated community-onset (HACO) cases were those for which cultures were collected from outpatients or ≤2 days after hospital admission from patients with 1) a history of hospitalization (including admissions >24 hours' duration), surgery, dialysis, or residence in a long-term care facility in the year before the culture date, or 2) a central venous catheter present at the time of culture. Patients with community-associated (CA) cases had none of the HO or HACO risk factors listed above.

Connecticut is divided into 169 towns. Large towns were defined as those with a population ≥100,000 (n = 5);

medium-sized towns had a population of 50,000–99,999 as estimated in 2005 (n = 12); and small towns had a population <50,000 (n = 152). Connecticut has 32 acute care hospitals. Hospital volume was categorized according to total annual number of Connecticut resident bed-days in 2009 as reported to the Connecticut Office of Health Care Access. High-volume hospitals (n = 7) averaged >80,000 patient bed-days per year and included both Connecticut tertiary care hospitals; medium-volume hospitals (n = 12) averaged 40,000–79,999; and low volume hospitals (n = 13) averaged <40,000.

### Laboratory Methods

From April 1, 2005, through December 31, 2010, a sample of MRSA isolates from blood was systematically collected from 8 sentinel hospital laboratories (2 high-volume, 6 medium-volume hospitals). These laboratories represented 4 metropolitan areas. Isolates were only collected from specimens from case-patients who resided in towns in which >90% of residents received health care at 1 of the 8 hospitals. In 3 metropolitan areas, all available blood isolates were collected. In the fourth area, with both high-volume participating hospitals, collection was limited to 3 blood isolates per month from each laboratory.

Isolates were submitted to the Connecticut Department of Public Health Laboratory for confirmation of *S. aureus* by performing a catalase test using 3% hydrogen peroxide, followed by a tube coagulase test using rabbit plasma with EDTA (Becton Dickinson Microbiology Systems, Franklin Lakes, NJ, USA). If the tube coagulase result was equivocal, a rapid latex kit (Remel Products, Lenexa, KS, USA) was used to confirm the result. Kirby-Bauer disk diffusion with a cefoxitin disk was conducted to confirm methicillin resistance.

Isolates were subtyped by pulsed-field gel electrophoresis (PFGE) with the restriction endonuclease *Sma*I using the standard PulseNet method (*14*). PFGE patterns were electronically forwarded to CDC for analysis with BioNumerics version 4.01 (Applied Maths, Austin, TX, USA) and grouped into pulsed-field types by using Dice coefficients and 80% relatedness. Isolates with pulsed-field types USA300 (ST8-MRSA-4), USA400 (ST1-MRSA-4), USA1000 (ST59-MRSA-4), and USA1100 (ST30-MRSA-4) were considered community-related strains, per previous EIP protocol (*12*). Isolates with pulsed-field types USA100 (ST5-MRSA-2), USA200 (ST36-MRSA-2), and USA500–800 (ST8, ST45, ST72, and ST5-MRSA-4, respectively) were considered health care–related strains.

### Statistical Methods

Incidence rates by year were calculated by using US Census Bureau yearly population estimates, except for 2010, for which the 2010 census counts were used. Rates were calculated overall for each MRSA category and for strata defined by age and race/ethnicity within the categories. Rates for HO MRSA were also calculated by using hospital specific resident bed-days as reported to the Connecticut Office of Health Care Access for 2001–2009. For 2010, bed-days from 2009 were used because 2010 data were not available. One hospital was excluded from the bed-day analysis because that facility does not report patient bed-days to the Office of Health Care Access.

Analyses were performed using SAS version 9.2 (SAS Institute. Inc., Cary, NC, USA). Percentages of cases in different demographic categories were compared by using the $\chi^2$ test. Ten-year incidence trends were examined by using the $\chi^2$ test for linear trend. To examine changes in trends within the overall 10-year period, we analyzed trends by $\chi^2$ separately for 2001–2003, 2004–2006, and 2007–2010, in part because MRSA received substantial national publicity in 2007 after an article reported incidence at EIP sites (*12*), a high school student in Virginia died from MRSA (*15*), and many state legislatures passed laws mandating reporting of MRSA and/or hospital infections (*13*).

## Results

### Epidemiologic Findings

The incidence of MRSA overall and average annual incidence rates by each of the 3 categories are reported in Table 1. A total of 8,758 cases of MRSA infections were reported over the 10-year period, of which 920 (10.5%) were CA, 2,753 (31.4%) were HO, 5,075 (57.9%) were HACO, and 10 (0.1%) were undefined. The average annual incidence was 25.2 cases/100,000 population. Incidence sharply increased with age, with a >70-fold increase from 1.5/100,000 among children <18 years to 111.5/100,000 among adults $\geq$65 years ($p<0.00001$, $\chi^2$ for trend). The incidence in men was 1.6 times that in women. In addition, the incidence in non-Hispanic blacks was 1.4-fold higher than in whites, and the incidence in Hispanics was 0.64 that of whites. Furthermore, residents of large towns had 1.2 and 1.4 times the incidence of residents of medium-size and small towns.

The descriptive epidemiology of HO, HACO, and CA MRSA infections had similarities and differences. For each, incidence was higher with increasing age, and in men, blacks, and large town residents (Table 1). However, persons with CA MRSA tended to be younger (43.2% <50 years vs. 13.8% and 17.7% for HACO and HO, $p<0.00001$ for each), and CA was the only 1 of these 3 groups in which incidence in Hispanics was higher than in whites (4.1/100,000 vs. 3.0/100,000, $p = 0.01$).

The overall incidence of MRSA infections by year and the yearly rates for each of the 3 categories of MRSA are shown in Figure 1. All have statistically significant

Table 1. Cases and incidence rates of MRSA infection by place of onset and association with healthcare and demographic features, Connecticut, USA, 2001–2010*†

| Demographic characteristic | All MRSA | | HO | | HACO | | CA | |
|---|---|---|---|---|---|---|---|---|
| | No. | Rate | No. | Rate | No. | Rate | No. | Rate |
| Total | 8,758 | 25.2 | 2,753 | 7.9 | 5075 | 14.6 | 920 | 2.6 |
| Sex | | | | | | | | |
| M | 5,290 | 31.2 | 1,620 | 9.6 | 3,043 | 18.0 | 620 | 3.7 |
| F | 3,465 | 19.4 | 1,132 | 6.3 | 2,030 | 11.4 | 300 | 1.7 |
| Age group, y | | | | | | | | |
| <18 | 127 | 1.5 | 65 | 0.8 | 25 | 0.3 | 37 | 0.4 |
| 18–34 | 379 | 5.3 | 115 | 1.6 | 148 | 2.1 | 116 | 1.6 |
| 35–49 | 1,082 | 13.0 | 308 | 3.8 | 529 | 6.4 | 244 | 2.9 |
| 50–64 | 1,955 | 31.4 | 642 | 10.3 | 1088 | 17.5 | 222 | 3.6 |
| ≥65 | 5,205 | 111.5 | 1,619 | 34.7 | 3,279 | 70.2 | 301 | 6.4 |
| Race/ethnicity‡ | | | | | | | | |
| White, non-Hispanic | 4,649 | 25.6 | 1,338 | 7.4 | 2,765 | 15.2 | 537 | 3.0 |
| Black, non-Hispanic | 815 | 35.9 | 232 | 10.2 | 466 | 20.5 | 116 | 5.1 |
| Hispanic | 467 | 16.5 | 107 | 3.8 | 245 | 8.6 | 115 | 4.1 |
| Town size§ | | | | | | | | |
| Large | 1,930 | 31.6 | 597 | 9.8 | 1067 | 17.5 | 265 | 4.3 |
| Medium | 1,969 | 26.2 | 635 | 8.5 | 1,174 | 15.6 | 159 | 2.1 |
| Small | 4,841 | 22.9 | 1,511 | 7.1 | 2,826 | 13.3 | 496 | 2.3 |

*MRSA, methicillin-resistant *Staphylococcus aureus*; HO, hospital onset; HACO, health care–associated community onset; CA, community associated.
†Incidence rates = average annual number of cases per 100,000 group-specific population using 2005 estimated population as the denominator.
‡Race-ethnicity only determined for all categories from 2004–2010. Denominator is 2007 estimated population.
§Large >100,000 population; medium 50,000–99,999 population; small <50,000 population.

trends from 2001–2010 (p≤0.002): all MRSA (−14.2%), HO (−48.4%), and HACO (−3.8%) decreased while CA (+124.8%) increased. The trends were not constant over the entire 10 years, however. When 3-year intervals were considered (2001–2003, 2004–2006, and 2007–2010), the only statistically significant trends were decreases in all MRSA, HO, and HACO infections during 2007–2010 (all p≤0.04) and an increase in CA during 2004–2006 (p = 0.001). During 2007–2010, overall MRSA incidence dropped 18.8%, HO dropped 33.2%, and HACO dropped 12.8%. During 2004–2006, CA increased 64.7%. However, during 2007– 2010, CA decreased 12.7% (p = 0.30).

When we assessed which demographic groups were most and least affected by these changes during 2007–2010, we found the following. For all MRSA infections during 2007–2010, a decrease ≥10% occurred in each demographic group. For HO MRSA, ≥10% decreases occurred in all groups except blacks (−7.3%). For HACO, ≥10% decreases occurred in all groups except those <18 years (+1%) and those 18–34 years of age (+14.2%). For CA, all groups showed decreases, except persons ≥50 years (+1%) and small town residents (+6.4%).

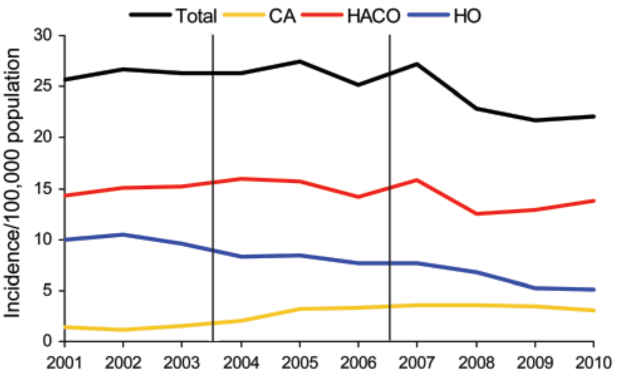


Figure 1. Incidence of methicillin-resistant *Staphylococcus aureus* infection, by relationship to healthcare and year, Connecticut, USA, 2001–2010. CA, community onset; HACO, health care–associated community onset; HO, hospital onset.

We also examined incidence of HO MRSA infections by hospital volume (Table 2). Increasing hospital volume was associated with higher average annual incidence (p<0.001). Overall, and for high- and medium-volume hospitals, incidence had a downward trend over the 10 years (p<0.0001 for each); the lowest rates were in 2009 and 2010 (Figure 2). No consistent 10-year trend was found for low-volume hospitals. When examined by the 3 periods (2001–2003, 2004–2006, 2007–2010), however, low-volume hospitals had increases in the first 2 periods (p = 0.04 and p = 0.07, respectively) and a decrease in the last (p = 0.01). Over time, rates based on hospital size have tended to converge, with a 3.9-fold difference in high-volume versus low-volume hospitals in 2001 reduced to 1.5-fold in 2010.

### Bacteriologic Findings

We examined the percentage of all MRSA isolates from the leading isolation sites by MRSA category. Blood isolates were most common (89.1%), followed by joint isolates (6.0%) and bone isolates (2.7%). CA infections were less likely than either HO or HACO infections to have a sterile-site isolate from blood (79.9% vs.88.2% and 91.2%, p<0.0001 for each), but more likely to have an isolate from a joint (18% vs. 2.0 and 5.9%, p<0.0001 for

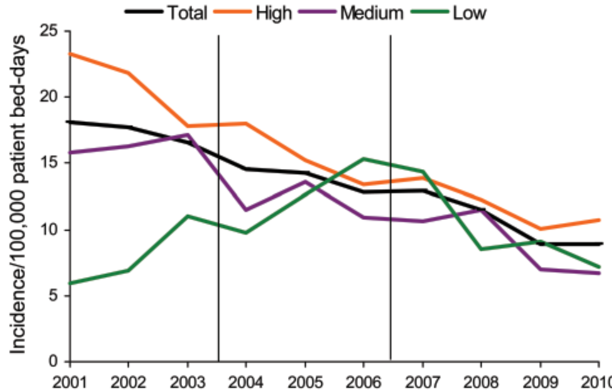


Figure 2. Incidence of hospital-onset methicillin-resistant *Staphylococcus aureus* infection, by hospital volume and year, Connecticut, USA, 2001–2010.

each). When 10-year trends were examined for each isolate site and place of onset category, 2 trends (at 2 sites) were significant. Joint isolates increased for HO and HACO MRSA infections, from 1.5% in 2001 to 5.4% in 2010 (p = 0.002) and from 4.7% in 2001 to 8.5% in 2010 (p = 0.005), respectively.

During 2005–2010, a total of 616 case-patients had blood isolates tested by PFGE at CDC; 609 had PFGE types in the range of USA100 to USA1100, inclusive. Of all PFGE types, 93 (15.3%) were community strains, of which USA300 was predominant (82, 88.2%). A total of 516 (84.7%) were health care strains, of which USA100 was predominant (456, 88.4%). There were 91 CA, 369 HACO, and 149 HO cases (Table 3). Isolates from CA cases were more likely than those from HACO and HO cases to have community PFGE types (49% vs. 11% and 6%, respectively), although the small difference in percentage between HACO and HO cases was statistically insignificant. During 2005–2010, isolates from CA and health care–associated (HACO and HO combined) cases were increasingly community PFGE types. The percentage of CA MRSA cases that were caused by community strain types increased from 28% to 56% (p = 0.02), and the percentage of health care–associated cases that were caused by community strains increased from 4% to 15% (p = 0.003).

To estimate trends in incidence of MRSA due to community and to health care strains, we applied the percentages of each to statewide incidence of CA and of health care–associated MRSA for each year during 2005–2010 (Table 4). For both CA and health care–associated MRSA, estimated incidence caused by community strains increased, while estimated incidence caused by health care strains decreased.

## Discussion

Invasive MRSA infections comprise at least 2 distinct MRSA groups: those caused by strains that evolved and spread in hospitals over the past 4 decades, and those caused by strains that evolved in the community and ≈15 years ago began to spread widely, causing community and institutional outbreaks of skin and soft tissue infections (*8*,*9*). Our population-based study analyzed trends in invasive MRSA in Connecticut from the health care and community perspectives. The key findings include the following: 1) the downward trend, previously documented in EIP sites during 2005 to 2008 (*16*), continued through 2010 in incidence of health care–associated invasive MRSA infections of both community and hospital onset; 2) invasive MRSA infection incidence declined consistently in hospitals of all sizes, in the community, and in almost all demographic groups since 2007; 3) community-associated invasive MRSA may have peaked; and 4) community strains are making an increasing contribution to invasive MRSA in hospitals and in the community.

At least 6 recently published large-scale studies have examined incidence trends in MRSA in the United States during various periods in the past decade (*16*–*21*). Most examined community-associated or community-onset MRSA and found large increases in the number of MRSA infections (*17*) or hospital admissions (*18*–*20*) from 1999–2000 through 2005–2007. The only published large-scale studies to find a trend toward decreasing incidence are 2 CDC studies. A study of invasive MRSA incidence in 9 EIP sites (including Connecticut) comprising ≈15 million persons, found that during 2005–2008, HO and HACO incidence declined 9.4% and 5.7%%, respectively (*16*). A more focused study of central line–associated bloodstream infections in US intensive care units found a net decrease of 49.6% during 1997–2007, with all the decrease occurring since 2001 (*21*).

The data from Connecticut during the same periods are consistent with those of other trends studies: CA MRSA was generally increasing from 2001 to 2007 (+158%), and HO and HACO MRSA decreased during 2005–2008

Table 2. Cases and incidence rates of hospital-onset MRSA infection by volume of hospital, at 29 acute care hospitals, Connecticut, USA, 2001–2010*

| Hospital volume† | No. cases‡ | Annual average incidence rate (range)§ |
|---|---|---|
| Total | 2,563 | 13.6 (8.9–18.1) |
| High | 1,494 | 15.5 (10.1–23.3) |
| Medium | 797 | 12.1 (6.7–17.1) |
| Low | 272 | 10.1 (5.9–15.3) |

*MRSA, methicillin-resistant *Staphylococcus aureus.*
†High, >80,000 patient bed-days/year; medium, 40,000–79,999 patient bed-days/year; low, <40,000 patient bed-days/year.
‡Number of cases is <2,753 because data were excluded from 3 (N = 32) hospitals that either did not report MRSA and/or the number of patient bed-days for some years.
§Annual average number of cases per 100,000 patient bed-days.

Table 3. MRSA sterile site isolates, by PFGE type, place of onset category, and year, Connecticut, USA, 2005–2010*

| MRSA category and PFGE type | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | Total | p value, trend |
|---|---|---|---|---|---|---|---|---|
| CA | | | | | | | | |
| C strain | 5 (28) | 7 (47) | 3 (30) | 10 (63) | 11 (69) | 9 (56) | 45 (49) | 0.02 |
| H strain | 13 (72) | 8 (53) | 7 (70) | 6 (38) | 5 (31) | 7 (44) | 46 (51) | |
| HACO | | | | | | | | |
| C strain | 5 (6) | 7 (9) | 4 (7) | 6 (11) | 7 (12) | 10 (21) | 39 (11) | 0.01 |
| H strain | 74 (94) | 67 (91) | 53 (93) | 49 (89) | 50 (88) | 37 (79) | 330 (89) | |
| HO | | | | | | | | |
| C strain | 0 | 0 | 2 (6) | 3 (11) | 4 (21) | 0 | 9 (6) | 0.07 |
| H strain | 45 (100) | 5 (100) | 30 (94) | 26 (90) | 15 (79) | 19 (100) | 140 (94) | |
| HA | | | | | | | | |
| C strain | 5 (4) | 7 (9) | 6 (7%) | 9 (11) | 11 (14) | 10 (15) | 48 (9) | 0.003 |
| H strain | 119 (96) | 72 (91) | 83 (83) | 75 (89) | 65 (86) | 56 (85) | 470 (91) | |

*Values are no. (%). MRSA, methicillin-resistant *Staphylococcus aureus*; PFGE, pulsed-field gel electrophoresis; CA, community-associated; HACO, health care–associated community onset; HO, hospital onset; HA, health care–associated (combined HACO and HO); C strain, community-related strain; H strain, health care–related strain.

(–20% each). The additional years of data in Connecticut help clarify the situation for hospital and health care–associated infections before and after the EIP study and the situation for CA infections since 2007. HO MRSA infections appeared to begin to decrease during2003–2004 and have remained on a downward trajectory since, while HACO infections appeared to be increasing during 2001–2004 and then began a generally downward trend. CA MRSA, after the large and generally continuous increase during 2001–2007, plateaued during 2007–2009 and then dropped 12.2% in 2010. The net effect of these varying trends on overall invasive MRSA infection has been an irregular, but net increase in, incidence during 2001–2007, and a large, persistent drop since 2007.

The typing data suggest that these varying trends in Connecticut can be connected. The subset of blood isolates typed during 2005–2010 showed that the percentages of all HO, HACO, and CA infections caused by community PFGE types are increasing, and those caused by health care–associated types are decreasing. When the percentages of community and health care–associated strains were applied to the statewide incidence of CA and of health care–associated MRSA for those years, statewide community-strain estimated incidence increased over time in each category, while statewide health care–associated strain estimated incidence decreased in each. Thus, the decrease in invasive MRSA infection can likely be explained by a reduction in disease incidence with health care–associated strains, while disease and, presumably, prevalence of community strains continue to increase.

Although our surveillance data analysis cannot directly address why health care strains and related invasive disease decreased while community strains and related disease did not, several explanations are possible. Since 2006, Connecticut laws have been passed that required the reporting of hospital infections and MRSA control policies in hospitals (*22*,*23*). In addition, in 2007, the Journal of the American Medical Association published a highly publicized article describing the magnitude and public health importance of health care–associated MRSA (*12*), which included Connecticut data, and the death of a Virginia high school student (*15*) generated substantial local as well as national media coverage. Collectively, these events likely created a high level of awareness among hospitals regarding MRSA control and increased attention to preventive efforts such as screening, isolation, and handwashing. As a consequence, carriage of MRSA in patients discharged from the hospital, mostly health

Table 4. Estimated incidence of MRSA, by category and strain PFGE type and year, Connecticut, USA, 2005–2010*†

| MRSA category and PFGE type | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | Incidence difference, 2005–2010 |
|---|---|---|---|---|---|---|---|
| CA | | | | | | | |
| Statewide incidence | 3.25 | 3.36 | 3.53 | 3.54 | 3.47 | 3.08 | –0.17 |
| % C strain‡ | 27.8 | 46.7 | 30.0 | 62.5 | 68.8 | 56.3 | |
| C strain incidence | 0.90 | 1.57 | 1.06 | 2.21 | 2.39 | 1.73 | +0.83 |
| H strain incidence | 2.35 | 1.79 | 2.47 | 1.33 | 1.08 | 1.35 | –1.00 |
| HA | | | | | | | |
| Statewide incidence | 24.16 | 21.78 | 22.54 | 19.27 | 18.13 | 18.95 | –5.21 |
| % C strain | 4.0 | 8.9 | 6.7 | 10.7 | 14.5 | 15.2 | |
| C strain incidence | 0.97 | 1.93 | 1.52 | 2.06 | 2.62 | 2.87 | +1.90 |
| H strain incidence | 23.19 | 19.85 | 21.02 | 17.21 | 15.51 | 16.08 | –7.11 |

*MRSA, methicillin-resistant *Staphylococcus aureus*; PFGE, pulsed-field gel electrophoresis; CA, community associated; C strain, community-related strain; H strain , health care–related strain; HA, health care–associated (combined hospital-associated community onset and hospital onset).
†Incidence per 100,000 population.
‡Percentage based on isolate testing shown in Table 3.

care–related strains, likely decreased, with subsequent reductions in health care–associated strain infections and in community infections resulting from community transmission of these strains.

In contrast, controlling community strains that occur outside the hospital is not as easy. Although proactive control efforts in institutions, including correctional facilities and sports facilities, should minimize the potential for institutional outbreaks, much community transmission occurs outside such settings. Thus, one could expect the sustained prevalence and continued transmission of community strains in the community with regular introduction in proportion to their incidence into health care settings and that their proportion of all MRSA infections would increase.

Although our data show a plateauing of CA MRSA, they also suggest that the increasing incidence of infection with community strains, mostly USA300, in Connecticut, is not over. The plateau effect appears to be because the rate of decrease in infections caused by health care strains is now equal to the rate of increase in community strains. Future trends will be determined in part by the relative dynamics of these MRSA strains.

Our study has several strengths: in particular, the surveillance is population-based, complete, enhanced by regular audits of all Connecticut laboratories for MRSA isolates, and of 10 years' duration. In addition, strain typing was performed on a subset of blood isolates.

However, this study also has several limitations. First, some HACO cases may have been misclassified as CA because only the most recent medical record was reviewed, and patients were not interviewed. Second, our data could have been influenced by trends in obtaining blood cultures from patients with fever, although we do not know whether a trend exists toward obtaining fewer blood cultures on acutely ill, febrile patients, most of whom need hospitalization. Third, the sample of isolates characterized was not random and may not be representative of the state as a whole. However, it was a systematic sample obtained from 4 metropolitan areas that had a collective MRSA epidemiology mirroring that of the state as a whole. Fourth, the hypothesis that the sharp drop in health care–related MRSA incidence beginning in 2008 may be due to a combination of hospital and public awareness raised in 2006 and 2007 is based on ecologic information only. We have not systematically reviewed hospital screening, isolation, hand washing, and other relevant policies and practices to determine whether they changed before MRSA began to decrease in each hospital. Fifth, CA-MRSA strains mainly cause skin and soft tissue infections. Although overall invasive disease appears to be decreasing, the replacement of health care–associated MRSA strains with community strains could result in an increase in noninvasive infections. Finally, the Connecticut experience may not be generalizable to other states or geographic regions with potentially different MRSA dynamics.

In summary, after 7 years of stable to increasing incidence, HA, HO, and CA MRSA all have been decreasing since 2007, coincident with increased public, public health, and hospital attention. The decrease appears to be entirely due to a decrease in incidence of infections caused by health care–related strains of MRSA. Continued monitoring is needed to assess the sustainability of the apparent prevention gains.

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Dr Hadler is an associate professor at the Yale School of Public Health and senior epidemiologist with the Connecticut Emerging Infections Program. His primary research interests are conducting surveillance for, and reducing occurrence of, emerging infections of public health importance and describing disparities in occurrence using socioeconomic indicators.

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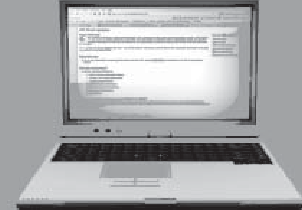

# Molecular Epidemiology of Geographically Dispersed *Vibrio cholerae*, Kenya, January 2009–May 2010

**Ahmed Abade Mohamed, Joseph Oundo, Samuel M. Kariuki, Hamadi I. Boga, Shanaz K. Sharif, Willis Akhwale, Jared Omolo, Anyangu S. Amwayi, David Mutonga, David Kareko, Mercy Njeru, Shan Li, Robert F. Breiman, and O. Colin Stine**

Numerous outbreaks of cholera have occurred in Kenya since 1971. To more fully understand the epidemiology of cholera in Kenya, we analyzed the genetic relationships among 170 *Vibrio cholerae* O1 isolates at 5 loci containing variable tandem repeats. The isolates were collected during January 2009–May 2010 from various geographic areas throughout the country. The isolates grouped genetically into 5 clonal complexes, each comprising a series of genotypes that differed by an allelic change at a single locus. No obvious correlation between the geographic locations of the isolates and their genotypes was observed. Nevertheless, geographic differentiation of the clonal complexes occurred. Our analyses showed that multiple genetic lineages of *V. cholerae* were simultaneously infecting persons in Kenya. This finding is consistent with the simultaneous emergence of multiple distinct genetic lineages of *V. cholerae* from endemic environmental reservoirs rather than recent introduction and spread by travelers.

Cholera, caused by the bacterium *Vibrio cholerae* and characterized by a profuse watery diarrhea, has been a serious public health problem since the first recorded pandemic in 1817. In 2009, the World Health Organization (WHO) reported just over 220,000 cholera cases and nearly 5,000 cholera-related deaths in 45 countries; however, these estimates are thought to be substantially underestimated because many countries where cholera is endemic do not report cases. During the past 20 years, the highest reported incidence shifted from the Americas to Africa. Africa accounted for 98% of reported cholera cases and 99% of reported cholera-related deaths during 2009.

Kenya has had numerous outbreaks of cholera since the first case was detected there in 1971 (*1*); 15 discrete outbreaks of cholera were documented during 1971–2010 (*2*–*6*). In western Kenya during January–April 2008, a cholera outbreak affected 10 administrative districts in Nyanza Province (adjacent to Lake Victoria), resulting in 790 cases and 53 deaths (case-fatality rate 6.7%) (*6*). The peak of the outbreak (January 2008) occurred after the December 2007 presidential election in Kenya, which had disputed results that triggered periods of protest, violence, public transportation disruption, and work stoppages throughout the country.

After an outbreak subsides in the lake region of Kenya, it has been suggested that *V. cholerae* becomes extinct in that locale but that isolated pockets of disease linger elsewhere in the region, and when the climate becomes favorable, *V. cholerae* reemerges and spreads from the refuge (*7*). The most recent outbreak occurred during January 2009–May 2010; cholera was detected in at least 52 districts throughout the country, and a total of 11,769 cases and 274 deaths (case-fatality rate ≈2.3%) were reported to the Kenya Ministry of Public Health and Sanitation. The regularity of these outbreaks indicates that *V. cholerae* might be frequently spread by travelers or that it is endemic to the area.

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Published epidemiologic studies used the best methods available at the time to differentiate isolates of *V. cholerae* in Kenya. For example, several investigations (*8*) used pulsed-field gel electrophoresis (PFGE) to characterize the genetic relatedness of the isolates responsible for cholera outbreaks. These studies found that all of the isolates were closely related, as would be expected if there was a spread from a single source. However, PFGE (*9,10*) discriminates poorly among serotype O1 and O139 *V. cholerae* strains. Multilocus-variable tandem repeat analysis (MLVA), however, has been reported to be useful in differentiating *V. cholerae* O1 strains in various rural communities and within households (*11–14*). To more fully understand the epidemiology of cholera in Kenya, we used MLVA to characterize the genetic relatedness of *V. cholerae* strains isolated from persons throughout the country.

## Materials and Methods

### Study Regions

We identified all districts in Kenya that reported cases of cholera during January 2009–May 2010. The Division of Disease Surveillance and Response, Ministry of Public Health and Sanitation, Kenya, provided a list of cases that met a clinical case definition for cholera. For the purpose of this study, we defined cholera as sudden onset of ≥3 episodes of watery diarrhea in a 24-h period in a person ≥2 years of age (compared with 5 years of age in the WHO definition) or <2 years of age if a clinician suspected cholera and *V. cholerae* was isolated from a stool specimen (corresponds with the WHO confirmed case definition, http://www.who.int/cholera/technical/prevention/control/en/index1.html). We used records from the provincial headquarters of the affected provinces and districts to update the list. After compiling a complete and up-to-date national list, we divided the country into 5 geographic regions, according to local climate conditions: the coastal region, the arid and semi-arid region, the lake region, the lower eastern region, and the highland region (Figure 1). We estimated the population of these regions by adding the population of districts within each region (data provided by the National Bureau of Statistics, Kenya 2009 Population and Housing Census Highlights, www.knbs.or.ke/Census%20Results/KNBS%20Brochure.pdf).

### Isolation of *V. cholerae* Strains in Kenya

Stool or rectal swab specimens were obtained from 222 persons with suspected cases of cholera who met the clinical case definition during the 2009–2010 cholera outbreak. The specimens were transferred onto Cary-Blair medium and transported (at 2°–8°C) to the Kenya National Public Health Laboratory Services, Nairobi, within 48 hours after collection. They were then cultured (37°C, 8 h) in alkaline peptone water and subcultured (37°C, 18–24 h) on thiosulfate–citrate–bile salts–sucrose agar (HiMedia Laboratories Ltd., Mumbai, India). After the specimens were subcultured, putative *V. cholerae* isolates were examined for sucrose fermentation. Suspicious colonies were subcultured (37°C, 18–24 h) again on Mueller-Hinton agar (Scharlau Chemie, Barcelona, Spain). All isolates were tested for the presence of oxidase, and they were serotyped with a polyvalent O1 antiserum and with monospecific Inaba and Ogawa antisera (Denka Seiken, Tokyo, Japan). All confirmed *V. cholerae* isolates were stored at −80°C in trypticase soy broth (Scharlau Chemie) supplemented with 20% (vol/vol) glycerol.

### Genotyping

Of the 222 stool specimens collected, 173 (78%) yielded *V. cholera* isolates; they were stored at −80°C until use. Of these 173 frozen isolates, 170 were revived by streaking onto Luria-Bertani agar and grown overnight at 37°C. Single, well-isolated colonies were selected to be grown in Luria-Bertani broth overnight at 37°C. We isolated *V. cholerae* DNA from the broth cultures by using PrepMan Ultra (ABI, Foster City, CA, USA) according to the manufacturer's instructions. We used PCR and primers as described (*13*) to amplify 5 loci containing variable length tandem repeats. Agarose gel electrophoresis was used to confirm the presence of amplified products.

The fluorescent-labeled products were separated and detected by using a model 3730xl Automatic Sequencer (ABI); internal lane standards (Liz600; ABI) and the GeneScan program (ABI) were used to determine produce sizes. Genotypes were determined by using published formulas to calculate the number of repeats from the length of each allele and to order the alleles at the 5 loci. The 5 loci, in order, are VC0147, VC0436–7 (intergenic), VC1650, VCA0171, and VCA0283; thus, the genotype 9,4,6,19,11 indicates that the isolate has alleles of 9, 4, 6, 19, and 11 repeats at the 5 loci, respectively. These standard loci will be in the global *V. cholerae* MLVA database, which is currently being developed (contact jimmyloh@dso.org.sg). Relatedness of the strains was assessed by using eBURSTv3 (http://eburst.mlst.net). Genetically related genotypes were defined as those possessing identical alleles at 4 of the 5 loci. The mismatch amplification mutation PCR was used to screen for the cholera toxin–encoding gene, as described (*15*).

## Results

### Epidemiology

During January 2009–May 2010, 11,769 cases of cholera were reported to the Division of Disease Surveillance and Response, Kenyan Ministry of Public Health and Sanitation. Demographic and geographic

Figure 1. Geographic/climatic regions as defined in a study of the genetic relatedness of O1 *Vibrio cholerae* isolates, Kenya, January 2009–May 2010.

information was obtained for 10,497 of the case-patients, of whom 246 (2.3%) died and 173 (1.65%) had laboratory-confirmed cholera. Case-patients who met the clinical case definition were reported from 52 (35%) of the country's 149 districts and from all 5 geographic regions (Table 1). The age range for case-patients was 1–76 years (overall mean 23 years, SD ± 18 years). The attack rate ranged from 0.02% in the lake and highland regions to 10-fold higher (0.2%) in the arid and semi-arid region. Case fatality rates ranged from 0.7% in the coastal region to 4.0% in the arid and semi-arid region (Table 1).

Cases of cholera were reported almost every day during January 2009–May 2010, and the number of cases reported during any 1 day ranged from 1 to 160 for the entire country. The first reported case was from the lake region (reported on January 2, 2009), and index cases appeared during the next 2 months in each of the other 4 regions. In the highland (including Nairobi), arid and semi-arid North, lower eastern, and coastal regions, the index cases were reported on February 22, February 26, March 12, and May 28, respectively. Every index case heralded the start of an apparent unified outbreak in the respective regions (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-1774-Techapp.pdf), during which time, the number of cases progressively increased. For example, in the lake, arid and semi-arid, highland, and lower eastern regions, the first outbreak peak occurred on January 20, April 7, May 5, and November 4, respectively. After the initial flush of cases in each region, the number of cases per week decreased to few or none; however, additional peaks, with numerous cases per week, would occur later.

## Genetic Relatedness

### Classical ctxB, Biotype and Serotype

Consistent with the possible spread of *V. cholerae* across the country, each of the 170 isolates was biotype El Tor and carried the classical *ctxB* allele, as measured by mismatch amplification mutation PCR. In contrast, 84% of the isolates were serotype Inaba, and the other 16% were serotype Ogawa.

### Multilocus-Variable Tandem Repeat Analysis

MLVA of the *V. cholerae* O1 isolates revealed extensive genetic diversity. To determine whether the strains were genetically related, we genotyped all 170 isolates at 5 loci containing variable numbers of tandem repeats. All loci exhibited substantial variation; for example, VC0147, VC436–7 (intergenic), VC1650, VCA0171, and VCA0283 had 9, 4, 6, 21, and 24 alleles, respectively. When each isolate was assigned a genotype (on the basis of and in order of the number of repeat units at each locus), 106 genotypes were detected among the 170 isolates. eBURSTv3 analysis to determine the genetic relatedness of the genotypes revealed 5 clonal complexes, each comprising a series of genotypes that differed by an allelic change at a single locus. In addition, we detected 19 singleton genotypes that were unrelated to any other genotype; that is, they differed at ≥2 loci from all other genotypes. The arid and semi-arid region yielded 42% (8 isolates) of these singletons; the coast, highland, and lake regions contributed 26% (5 isolates), 21% (4 isolates), and

Table 1. Cholera attack rate and CFR by geographic/climatic region, Kenya, January 2009–May 2010*

| Region | No. cases | Mean age ± SD, y (range) | Attack rate, %† | No. deaths (CFR, %) |
|---|---|---|---|---|
| Coastal | 1,484 | 21 ± 18 (1–70) | 0.07 | 10 (0.7) |
| Highland | 1,139 | 28 ± 18 (1–75) | 0.02 | 46 (4.0) |
| Arid and semi-arid | 4,210 | 25 ± 19 (1–70) | 0.20 | 146 (3.5) |
| Lake | 1,019 | 25 ± 17 (1–76) | 0.02 | 23 (2.3) |
| Lower eastern | 2,645 | 25 ± 18 (1–67) | 0.12 | 21 (2.3) |
| Total | 10,497 | 23 ± 18 (1–76) | 0.07 | 246 (2.3) |

*CFR, case-fatality rate.
†Denominator was based on population as provided by the National Bureau of Statistics (Kenya 2009 Population and Housing Census Highlights, www.knbs.or.ke/Census%20Results/KNBS%20Brochure.pdf).

11% (2 isolates), respectively. No singletons were detected in the lower eastern region.

The 3 largest clonal complexes (designated 1, 2, and 3) occurred throughout most of the country. Clonal complex 1 contained 52 different genotypes among 89 isolates (Figure 2, panel A), and it was observed in every region (Table 2). The most common genotype, identified as the founder genotype (defined as the genotype that differed from the largest number of other genotypes at only 1 locus), was observed in 22 isolates collected from informal settlements around Nairobi. The founder genotype radiated into 12 other genotypes, and 7 of those differentiated further. No distinct correlation between the geographic locations and genotype of the isolates was detected.

Clonal complex 2, which contained 20 different genotypes among 33 isolates (Figure 2, panel B), was detected in the Rift Valley (in the western part of the arid and semi-arid region) and in the coastal, lower eastern, and highland (primarily represented by informal settlements around Nairobi) regions. Clonal complex 3, which contained 11 genotypes among 23 isolates (Figure 2, panel C) was detected in the coastal, lake, and highland regions and in the Rift Valley (arid and semi-arid region).

Some geographic differentiation of the clonal complexes appears to have occurred (Table 2). The 3 large clonal complexes were distributed in a statistically significant ($p<0.0002$, by $3 \times 4$ $\chi^2$ test), nonrandom manner across the arid and semi-arid, coastal, highland, and lower eastern regions (the lake region had too few isolates to be tested). This significant difference can be attributed to the finding that clonal complex 2 was the most common complex in the arid and semi-arid region, and clonal complexes 1 and 3 were the most common complexes in the highland region. These findings are consistent with our observation that clonal complexes 4 and 5 were each seen in only 1 region, but they are very small groups. Clonal complex 4 isolates were found during various months; however, the complex 5 isolates were found only during February 2009, which was during the beginning of the outbreak in Moyale, an isolated village on the border with Ethiopia.

Figure 3 shows the temporal distribution of the 5 genetically distinct clonal complexes and the singletons for 4 regions; no temporal separation of the various clonal complexes is apparent. Isolates collected in February 2009 from the highland region and arid part of the arid and semi-arid region belong to distinct clonal complexes, 1 and 5, respectively; however, isolates collected in March 2009 from the same 2 regions all belong to clonal complex 1. Substantial variation was observed early in the outbreak: 2 or 3 clonal complexes are represented in the first 7 isolates genotyped from each region. In the highlands region, every 3-month period in which isolates were found has ≥2 distinct genotypes (Figure 3, panel A). In the coastal region, every month >1 isolate assayed has ≥2 genotypes (Figure 3, panel B). In the arid and semi-arid region, April and May 2009, when 4 isolates were assayed, are the only 2 consecutive months with a single genotype (Figure 3, panel C). Even in the lower eastern region, where only 13 isolates were assayed, multiple genotypes were observed in the 2 months in which >1 isolate was found (Figure 3, panel D).

## Discussion

The results of our genetic analyses suggest that the most recent outbreak of cholera in Kenya (beginning in

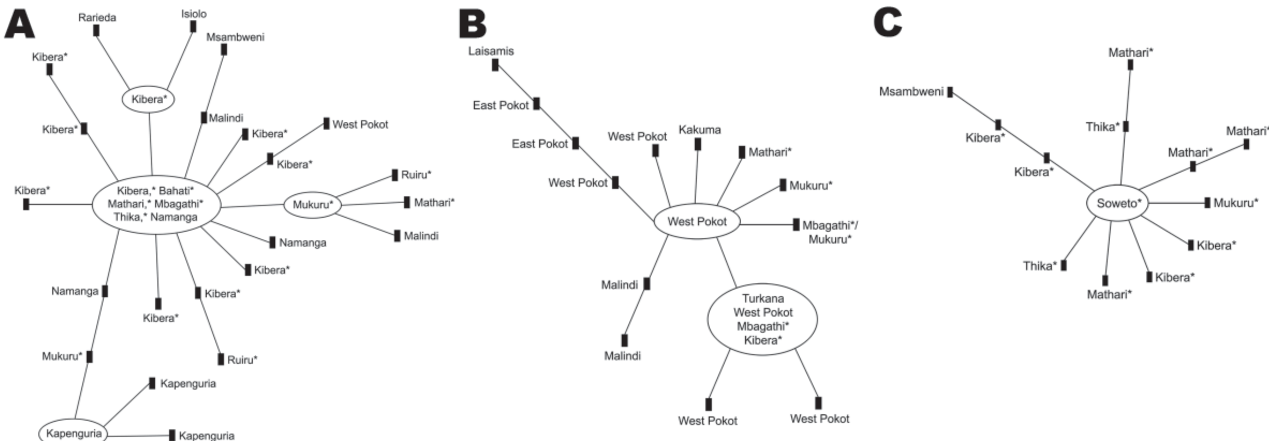


Figure 2. Genetic relatedness of O1 *Vibrio cholerae* isolates from an outbreak of cholera, Kenya, January 2009–May 2010. The 3 largest clonal complexes are shown; smaller clonal complexes consisted of 2 or 3 genotypes. A) Clonal complex 1 was observed in each geographic/climatic region. B) Clonal complex 2 was detected in the Rift Valley (western part of the arid and semi-arid region) and in the coastal, lower eastern, and highland (primarily in informal settlements around Nairobi, represented by asterisks) regions. C) Clonal complex 3 was detected in the coastal, lake, and highland regions and in the Rift Valley (arid and semi-arid region).Genetic relatedness was determined by using eBURSTv3 (http://eburst.mlst.net/). Each genotype is represented by a node in the diagram; each connecting line represents an allelic change at a single locus.

Table 2. Number of *Vibrio cholerae* isolates, by clonal complex and geographic/climatic region, Kenya, January 2009–May 2010

| Region | Clonal complex, no. isolates | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | Singletons |
| Arid and semi-arid | 17 | 20 | 4 | 0 | 2 | 8 |
| Highland | 50 | 8 | 17 | 2 | 0 | 4 |
| Lower eastern | 7 | 5 | 0 | 0 | 0 | 0 |
| Coastal | 10 | 2 | 1 | 0 | 0 | 7 |
| Lake | 1 | 0 | 1 | 0 | 0 | 2 |
| Total | 85 | 35 | 23 | 2 | 2 | 21 |

January 2009) represented several distinct genetic lineages of *V. cholerae* that emerged simultaneously, perhaps facilitated by environmental and behavioral factors around the country. Thus, these data suggest that these outbreaks likely resulted from endemic foci rather than from recent introduction and spread by travelers.

In contrast, previous outbreaks of cholera in Kenya have been attributed to the spread of *V. cholerae* by travelers. Outbreaks in the 1990s and in 2005 were attributed to a clone carried by travelers to Nairobi; from there, it spread to other locations in the country (*4*). Likewise, the well-publicized 2010 cholera outbreak in Haiti also appears to have resulted from the introduction of *V. cholerae* by travelers (*16*). In addition, there have been repeated introductions of *V. cholerae* into previously disease-free locations by travelers from Southeast Asia and other areas where cholera is endemic (*17*). On a smaller scale, *V. cholerae* is thought to persist around the African Great Lakes Region because, although it becomes extinct in areas, it is still present in other localities and can spread when weather conditions are favorable (*7*).

Genetic uniformity is expected when *V. cholerae* is spread from a single source. This expectation conflicts with the observation that isolates with Inaba and Ogawa serotypes occurred during the outbreak. However, the expectation is consistent with the genetic evidence that all isolates had the El Tor biotype and identical (by Tenover's criteria) (*18*) PFGE patterns (J.O. Oundo, et al. unpub. data). The nearly identical (single band) differences in the PFGE patterns are consistent with whole genome sequencing results showing that the toxigenic El Tor lineage of O1 *V. cholerae* has few large (several kilobytes) insertions or deletions (*19*) and few nucleotide changes (*17*). Large insertions or deletions or nucleotide changes in restrictions sites are the mutations that produce altered PFGE patterns; their absence in the El Tor lineage is consistent with the minimal number of PFGE patterns.

All *V. cholerae* strains that we studied from the 2009–2010 outbreak in Kenya had the classical *ctx*B allele, which is consistent with a clonal origin. The earlier population of *V. cholerae* is presumed to have contained only the El Tor allele because the classical *ctx*B allele was not detected before 1989 in isolates with the El Tor background (*20*). Thus, the presence of the classical allele in all isolates from the 2009–2010 outbreak most likely represents a rapid shift in allele frequency. Such a shift occurred in O1 *V. cholerae* in Kolkata, India, when allele frequency changed from 100% El Tor in 1989 to 100% classical *ctx*B in 1995 (*20*). It is of critical clinical importance that, the classical *ctx*B allele, when found in the background of an El Tor strain, has been associated with a more severe form of cholera than that caused by a strain with the El Tor *ctx*B allele (*21,22*).

Our MLVA genotyping results for 170 *V. cholerae* isolates from the 2009–2010 outbreak in Kenya showed extensive genetic diversity: we found 5 clonal complexes and 19 singleton genotypes. In contrast, studies using PFGE found genetic uniformity among *V. cholerae* isolates from

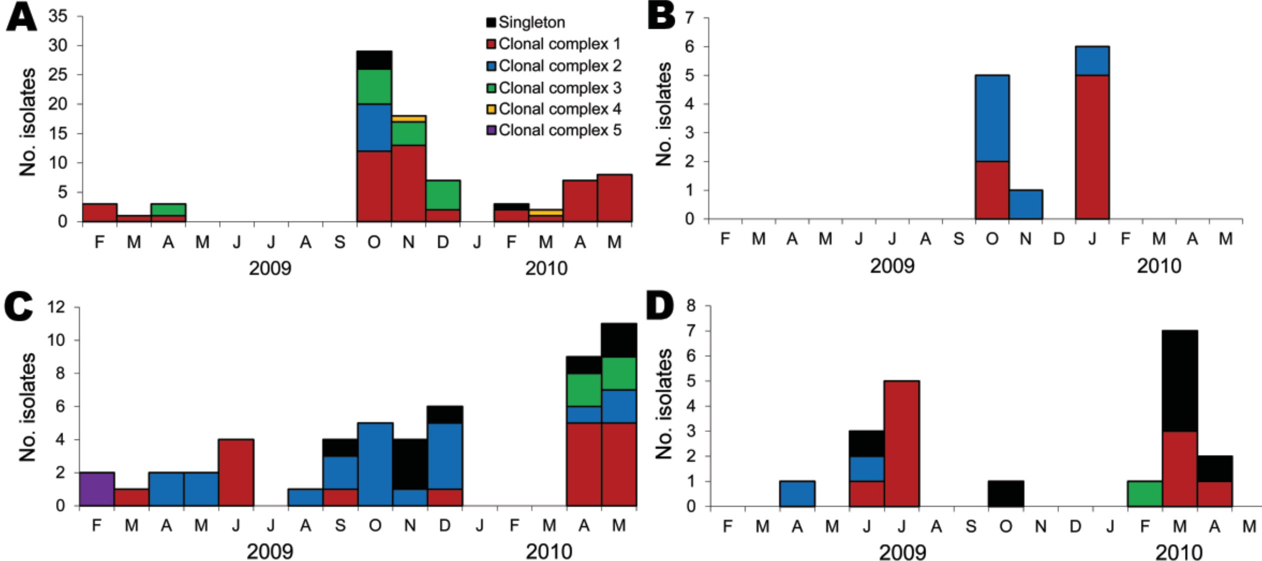


Figure 3. Distribution of *Vibrio cholerae* isolates, by clonal complex and month of isolation, Kenya, January 2009–May 2010. A) Highland region, including informal settlements around Nairobi. B) Coastal region. C) Arid and semi-arid region. D) Lower eastern region.

1994 to 2010 (*5,8*) (J.O. Oundo, et al. unpub. data). These differences may be explained by the finding that MLVA is superior to PFGE for discriminating between isolates of *V. cholerae* O1 (*11–13*).

An alternative explanation for the substantial variation observed among *V. cholerae* isolates from Kenya is that their tandem repeat loci may result from a rapid accumulation of mutations. However, 3 findings have documented the relative stability of those loci. First, an analysis of the variation occurring during a month-long serial passage revealed that the 3 large chromosomal loci (the primary determinants of membership in the various clonal complexes) were largely stable (*13*). Second, the clonal complexes remained distinct during 3 consecutive years in Bangladesh (*13*), longer than the 17 months of the 2009–2010 outbreak in Kenya. Third, all isolates from the cholera outbreak that began in October 2010 in Haiti belonged to a single clonal complex (*16*). These observations and the co-occurrence of distinct clonal complexes are consistent with the idea that there were multiple simultaneous outbreaks of cholera in Kenya.

Diverse MLVA genotyping results are found in countries where *V. cholerae* is endemic. In India, 6 clonal complexes were detected in *V. cholerae* O1 isolates (*23*), and in Bangladesh, 7 clonal complexes were detected (*12*). Consistent with these findings, we detected 5 clonal complexes in isolates from Kenya. The genetic diversity is also consistent with data from single-nucleotide polymorphism analyses that showed the several distinct waves of immigration of *V. cholerae* into Kenya (*17*), if the descendants of these immigrants settled and survived. The presence of multiple distinct lineages across the country supports the notion that there have been multiple introductions of *V. cholerae* and that over time these strains spread countrywide. The multiplicity of clonal complexes is in stark contrast to the apparent uniformity of the *ctx*B allele. An earlier study demonstrated that the *ctxB* alleles, as part of a mobile genetic element, were found in distinct locations in different MLVA clonal complexes (*14*).

The timing of the observed diversity is consistent with multiple separate outbreaks and not with the spread of disease by travelers. In February 2009, isolates from highland region, including Nairobi, belonged to clonal complex 1 and might have spread from the lake region. However, isolates from Moyale in the arid and semi-arid region belonged to clonal complex 5, and the time between the initial observations in the southern part of the country and the far north was too short for the isolates to have evolved into a new clonal complex. In addition, the far north is a region where travel is difficult and people from south seldom travel. Thus, these 2 outbreaks appear to be temporally, geographically, and genetically separate. As stated, the mutations necessary for 1 clonal complex to evolve into another are not expected to occur in a few months. Thus, during the 2009–2010 outbreak, the occurrence of isolates from a second complex in the highland region (clonal complex 3) and the arid and semi-arid region (clonal complex 2) cannot be explained by evolutionary changes. In addition, no source was found from which travelers could have introduced these *V. cholerae* strains. A similar argument can be made for clonal complex 4 isolates and for each of the singletons scattered across the country. That is, the time to their appearance was too short to represent an evolutionary change, and there was no source from which a traveler spread the genetically distinct isolates. Thus, our analyses led to the conclusion that *V. cholerae* is endemic in Kenya. The extensive genetic variation among the isolates exposes a limitation in our sampling scheme. Our sampling should have included many more isolates from each region and each time period. The observed genetic differences permit distinguishing between regions, but the small number of samples limits what can be inferred about what is happening within a region.

Kenya has experienced multiple major outbreaks of cholera, and different regions of the country have reported different attack rates. During the 2009–2010 outbreak, all of the isolates collected contained the classical *ctxB* allele. Despite this genetic uniformity, our MLVA results showed that the isolates had extensive genetic variation within and between geographic locations. The genetic relatedness studies we performed showed that 5 clonal complexes and 106 different genotypes were part of the outbreak; thus, *V. cholerae* had several genetic lineages. Our data are consistent with *V. cholerae* isolates being endemic throughout Kenya.

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# Community Survey after Rabies Outbreaks, Flagstaff, Arizona, USA

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Flagstaff, Arizona, USA, experienced notable outbreaks of rabies caused by a bat rabies virus variant in carnivore species in 2001, 2004, 2005, 2008, and 2009. The most recent epizootic involved transmission among skunk and fox populations and human exposures. Multiple, wide-ranging control efforts and health communications outreach were instituted in 2009, including a household survey given to community members. Although the Flagstaff community is knowledgeable about rabies and the ongoing outbreaks in general, gaps in knowledge about routes of exposure and potential hosts remain. Future educational efforts should include messages on the dangers of animal translocation and a focus on veterinarians and physicians as valuable sources for outreach. These results will be useful to communities experiencing rabies outbreaks as well as those at current risk.

More than 90% of rabies cases in the United States are in wild animals. Most reported cases of rabies occur among carnivores, including raccoons, skunks, and foxes, in addition to many bat species. Despite the elimination of canine rabies virus variants in the United States, domestic animals, including cats and dogs, are infected each year from exposures to rabid wildlife. In addition, ≈2–4 human rabies cases are reported each year in the United States (*1*), and exposure to rabid animals or animals suspected of being rabid is common, with ≈35,000–38,000 persons receiving rabies postexposure prophylaxis (PEP) each year (*1*,*2*). One of the primary methods for rabies prevention and control is practical and accurate public health information. Recognition of the signs and severity of rabies, exposure routes, behavioral and environmental risk factors, and appropriate domestic animal welfare are critical messages for disease prevention and require appropriate public education for persons of all ages (*3*,*4*).

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Rabies virus is generally transmitted among members of the same species, and specific rabies virus variants are associated geographically with independent reservoir species. Spillover of rabies virus variants from 1 species to another occurs, but sustained transmission of such variants in nonreservoir species is rare (*4*). The area around Flagstaff, Arizona (Coconino County), USA, was free of sustained rabies virus transmission until 2001, when a spillover of a bat rabies virus variant was followed by a suspected host shift, with increased transmission in striped skunk (*Mephitis mephitis*) populations (*5*). Control measures were launched to halt rabies spread in skunks and limit the potential for human exposures (*6*). These efforts appeared to control rabies spread in skunk populations until 2004, when 5 striped skunks and 1 gray fox (*Urocyon cinereorgenteus*) were diagnosed as rabid, and rabies was confirmed in an additional striped skunk, a gray fox, and a feral cat (*Felis catus*) in 2005 (*5*). Rabies was quiescent after this resurgence in 2004/2005 until fall 2008 when the disease was confirmed in several gray foxes and striped skunks (*4*). The establishment of rabies in fox populations was troubling because the extensive home range of foxes threatened its containment in the Flagstaff area. Given the size of this epizootic, the potential for spread to other areas, and several notable human exposures, a large, interagency effort was launched to control the resurgence of rabies in Flagstaff.

In October 2009, a survey was distributed to Flagstaff households in an area where rabid animals had been captured in 2008 and 2009. This area also had a history of rabies epizootics since 2001 (*5*,*6*). Attitudes and practices regarding management of exposure to domestic and wild animals are essential to define in areas where persons and their pets may have an increased chance of coming into contact with a rabid animal. An assessment of community knowledge of rabies and interactions with animal reservoirs can help target educational messages during seasonal disease peaks or at the beginning of an epizootic. We present an update on the most recent outbreak and the results of a community survey in Flagstaff.

## Methods

### Data Sources and Survey Design

Surveillance data for Coconino County of the numbers of rabid animals identified during 2000–2009, were obtained from the Arizona Department of Health Services. Emergency department admission data, in which the chief complaint included animal bites during 2005–2009, were obtained from the infection control office for the Flagstaff Medical Center.

Addresses of all households in the quarantine area of Flagstaff were provided by the Coconino County Public Health Services District. Occupancy status of the households was not available, and names associated with each address were permanently removed and not shared for the mailings. In October 2009, surveys were mailed to all households, and 1 adult from each household was asked to complete the survey. Respondents could complete the survey online or by an included paper-based form and returned in a prepaid envelope by mail. The surveys were anonymous and were not linked to a name or address. Educational material on rabies was not included, but for more information, respondents could request printed materials on a separate request form and were directed to the rabies website of the Centers for Disease Control and Prevention (CDC) (Atlanta, GA, USA) (*7*). The community survey was determined to be public health nonresearch by CDC. The survey elicited information from respondents on knowledge of rabies, the Flagstaff rabies outbreak, practices regarding domestic and wild animals, and adherance to quarantine restrictions. Survey questions are included in online Appendix Table 1 (wwwnc.cdc.gov/EID/article/18/6/11-1172-TA1.htm).

### Data Analysis

A general definition of "knowledge of rabies" was defined as the answer of "yes" when the respondent correctly identified that bites, scratches, and saliva were modes of rabies virus transmission and also identified 1 other incorrect mode of transmissions as a mode of transmission or did not identify any incorrect mode of transmission. A more specific definition of "knowledge of rabies in Flagstaff" was defined as "yes" if the respondent knew about the outbreak in Flagstaff and knew the 3 main animals which had rabies in Flagstaff (bat, skunk, and fox).

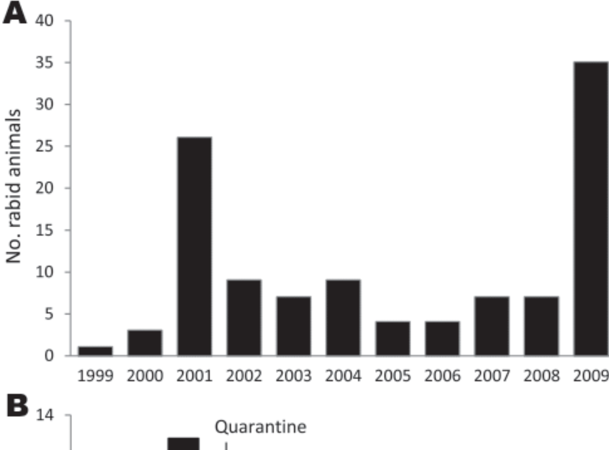
Univariate and multivariate analyses were conducted for 4 separate resident subpopulations to further characterize the groups. The outcomes of interest were 1) knowledge of rabies in Flagstaff, 2) pet owners, 3) dog owners, and 4) translocators (persons who trapped and moved wild animals on their property). Odds ratios with 95% CIs for individual characteristics were calculated by using logistic regression. Characteristics that were considered associated ($p<0.1$) with each outcome in the univariate analysis were further assessed through multivariate logistic regression models (*8*).

## Results

### Update on Rabies Epizootic

After a period of quiescence from 2005, another rabies epizootic occurred in Flagstaff during 2008 (Figure 1, panel A). Seven rabid animals were reported in Coconino County, including 2 foxes and 2 skunks (Table 1). No human exposures to rabid animals were reported in 2008, but several companion animal exposures occurred, including 6 cats exposed to rabid bats, 2 dogs exposed to rabid skunks, and 1 dog exposed to a rabid fox. In 2009, Coconino County reported 35 rabid animals. All rabies viruses typed from the immediate Flagstaff area (from 14 foxes and 1 ringtail cat) were identified as a bat rabies virus variant. Two human exposures to rabid animals were identified in 2009: one person had been bitten by a rabid fox and the other person had been bitten by a rabid skunk. Also, 3 companion dog exposures to rabid foxes were reported. A review of Emergency Department discharge data at Flagstaff Medical Center specified a total of 88 animal bite–related admissions during 2005–2009. In 2009, 25 animal bite–related admissions were recorded, and 12 (48%) persons received PEP.

The number of rabid animals and control measures that were initiated in Coconino County in 2009 are shown in Figure 1, panel B. During July 21–24, the US Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, distributed 140,000 oral rabies vaccination (ORV) baits containing a vaccinia–rabies glycoprotein vaccine (Merial, Duluth, GA, USA) by air and ground over a 191-$km^2$ area in Coconino County, targeting gray foxes (Figure 2). The vaccinia–rabies glycoprotein vaccine is not effective for vaccinating skunks against rabies (*9*). A 6-week trap, vaccinate, release campaign targeting skunks was initiated in the eastern portion of Flagstaff



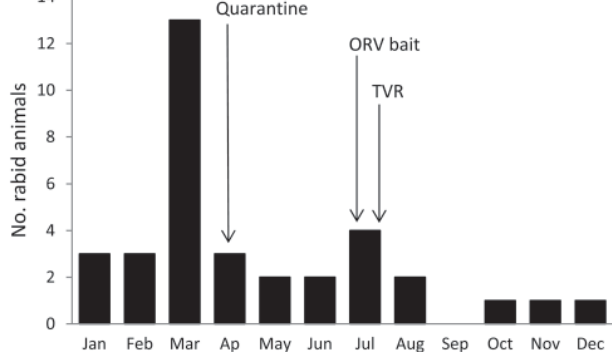




Figure 1. Reported rabid animals, Coconino County, Arizona, USA. A) Number of rabid animals confirmed by laboratory testing, 1999–2009. B) Number of rabid animals during 2009 and response activities. ORV bait, oral rabies vaccination bait; TVR, trap, vaccinate, release campaign.

at the end of July. Additional control initiatives included prohibiting relocation of nuisance wildlife, comprehensive public education on rabies, rabies vaccine clinics for pets, a leash policy for pets on trails, and quarantine. The quarantine was established from April 7 through September 13, 2009, for a 15-mile-radius area centered on Flagstaff and later expanded to the entire ORV zone. The following measures were mandatory: do not disrupt ORV baits, do not feed wild animals, enclose compost bins and piles, do not leave pet food outside after sundown, confine cats and dogs to an enclosure on the owner's property, keep pets on a leash when off of the owner's property, and maintain current rabies vaccination for cats and dogs.

Table 1. Annual number of rabid animals confirmed, Coconino County, Arizona, USA, 2008–2010

| Species | 2008 | 2009 | 2010 |
|---|---|---|---|
| Bat | 1 | 4 | 4 |
| Bobcat | 1 | 0 | 0 |
| Coyote | 0 | 1 | 0 |
| Fox | 3 (2*) | 24 (14*) | 0 |
| Ringtail | 0 | 1 (1*) | 0 |
| Skunk | 2 (2*) | 5 | 0 |
| Total | 7 (4*) | 35 (15*) | 4 |

*Number of animals positive for brown bat rabies virus variant.

### Demographic and Rabies-related Characteristics of Household Respondents

A total of 3,141 surveys were mailed, but 172 were returned because of an incorrect address or no occupancy of household, and 1,058 were completed and returned (35.6%); 1,039 written and 19 website-based. Most respondents had lived in Flagstaff for ≥10 years (74%), lived in Flagstaff year round (98%), were ≥51 years of age (68%), female (59%), and had at least a college degree (75%). A summary of responses is shown in online Appendix Table 1.

Most respondents recognized that rabies virus can be transmitted to humans from infected animals through a bite (97%), scratch (73%), or contact with saliva (74%). More than half of respondents thought rabies virus can be transmitted by contact with blood, almost a quarter by contact with an infected animal's urine or feces, and 13% identified skunk spray as infectious. Most residents were aware that skunks and foxes in Flagstaff may have rabies (89% and 73%, respectively), but only 52% were aware that bats in Flagstaff had rabies. Information about the current outbreak was ascertained by many methods, with newspapers or magazines being the most frequently cited source (78%).

Most (70%) respondents reported that if they were bitten or scratched by a domestic animal they would wash the wound with soap and water and likely seek medical care. More persons indicated they would seek medical care if they had an encounter with a wild (90%) animal than with a domestic (72%) animal. Most respondents indicated they would call one of 3 public agencies (city animal control, county health department, fish and game department) if they saw a sick animal than if they were bitten or scratched by an animal. Sixty residents (6%) reported seeing a sick wild animal on their property in the last 6 months. Of those that specified the type of animal, 21 persons (38%) reported a sick skunk, 20 (36%) a sick fox, and 1 (2%) a sick bat; 38% of responses were listed as "other." Thirty-six percent of the residents reported doing nothing after seeing the ill animal, while 36% called animal control, and 17% called the county health department.

Seventy-three respondents (7%) reported that they would trap and translocate a nuisance animal themselves. Ninety-five persons (9%) have personally relocated a wild nuisance animal that was on their property. Furthermore, 57% translocated the animals >5 miles from their property. Skunks were the most frequent animal to be translocated (56%).

Eighty-four percent of respondents were aware of the rabies quarantine, and 82% of those stated that they complied with quarantine restrictions. Twelve percent of

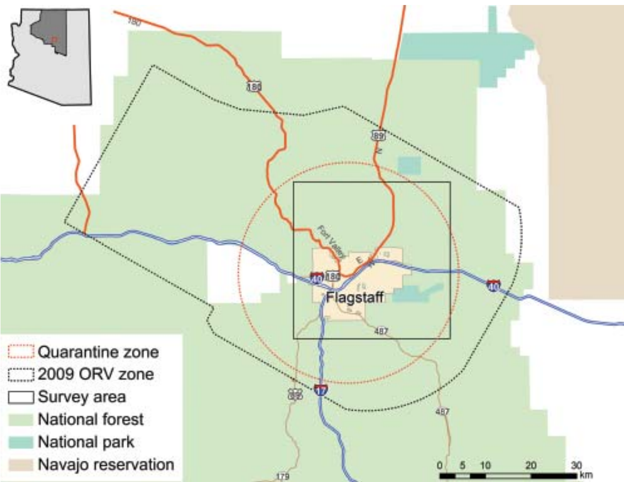



Figure 2. Flagstaff, Arizona, USA, survey area in relation to quarantine and oral rabies vaccination (ORV) zones.

respondents did not believe the requirement to keep pets on a leash at all times would help prevent rabies exposures.

One half of all households owned dogs, and 29% of all households owned cats (Table 2). The overall proportion of dogs vaccinated was 96%. A higher proportion of outdoor cats (90%) was vaccinated than were indoor (76%) or indoor/outdoor (84%), and the overall reported proportion of cats vaccinated was 81%.

### Rabies Knowledge in Flagstaff, Pet Ownership, Dog Ownership, and Translocation

Persons who had knowledge of rabies in Flagstaff were more likely to have knowledge of other aspects regarding rabies (online Appendix Table 2, wwwnc.cdc.gov/EID/article/18/6/11-1172-TA2.htm), including the quarantine and concern about rabies in Flagstaff; these characteristics were independently associated with knowledge of rabies in Flagstaff (Table 3). Male respondents were more likely to have knowledge of rabies in Flagstaff than female respondents. Multivariate analysis did not identify an independent association between those who have knowledge of rabies in Flagstaff and contact with a nuisance wild animal.

Pet and dog owners were more likely to have had contact with a sick domestic animal and to be aware of the rabies quarantine than those who did not own pets (online Appendix Table 2). Pet ownership in general was associated with knowledge of rabies. In multivariate analyses, pet owners were less likely to believe that the leash policy prevents exposures to rabid animals than non–pet owners. Dog owners were more likely to be aware of the 2009 quarantine (Table 3). There was significant interaction between age group and potential for contact with sick domestic animals, and for respondents ≤60 years of age, those that indicated potential for contact with a sick domestic animal were more likely to be general pet owners or dog owners. Among women, general pet owners were more likely to be aware of the quarantine than non–pet owners; this association was not seen for men. Men and women were similarly aware of the quarantine (83% and 84%, respectively).

Persons who had translocated nuisance animals were more likely to be male and to not own a pet (online Appendix Table 2), although these associations did not remain independently significant in the multivariate analysis (Table 3). In the multivariate analyses, those who moved animals from their property were more likely to have a potential for contact with a wild animal and to have lived in Flagstaff for at least 10 years.

## Discussion

An extensive outbreak control and education campaign took place in 2009. As observed in past interventions, the epizootic waned and in 2010 only 4 rabid bats were reported from Coconino County. This decline in rabid animals is likely attributable, in part, to the broad interagency control campaigns. Whether another epizootic will occur in Flagstaff remains to be determined. However, the multiple outbreaks over the last decade have resulted in a substantial change in rabies epizootiology in northern Arizona. The repercussions of a potential perpetuation of a bat rabies virus variant in gray fox populations are a concern, given the wide-ranging movements of these carnivores. In addition,

Table 2. Characteristics of households that owned pets, Flagstaff, Arizona, USA, 2009

| Characteristics | No. (%) |
|---|---|
| Pet owner | 684 (65)* |
| Dog owner | 528 (50)* |
| Dog(s) quarantined for possible rabies exposure | 21 (4) |
| No. dogs currently | |
| Mainly indoor | 638 (85) |
| Mainly outdoor | 115 (15) |
| Total | 753 (100) |
| No. dogs with current rabies vaccination | |
| Mainly indoor | 615 (96)† |
| Mainly outdoor | 106 (92)† |
| Total | 721 (96)† |
| Cat owner | 308 (29)* |
| No. cats currently | |
| Indoor | 261 (51) |
| Outdoor | 31 (6) |
| Indoor/outdoor | 218 (43) |
| Total | 510 (100) |
| No. cats with current rabies vaccination | |
| Indoor | 199 (76)† |
| Outdoor | 28 (90)† |
| Indoor/outdoor | 184 (84)† |
| Total | 411 (81)† |

*Frequency among all survey respondents (n = 1,058).
†Proportion vaccinated.

Table 3. Multivariate analysis of respondents' rabies knowledge in Flagstaff, pet ownership, dog ownership, and translocation, with demographic and rabies-related characteristics*

| Characteristic | Odds ratio (95% CI) | | | |
|---|---|---|---|---|
| | Knowledge of rabies in Flagstaff† | Pet owners | Dog owners | Translocators |
| Concern about rabies in Flagstaff | | | | |
| Concerned | 2.49 (1.21–5.15) | | | |
| Not concerned | | | | |
| Potential for contact with nuisance wild animal on property | | | | |
| Yes | | | | 16.33 (9.98–26.74) |
| No | | | | Referent |
| Aware of quarantine in Flagstaff during 2009 | | | | |
| Yes | 4.20 (2.67–6.62) | | 2.24 (1.55–3.23) | |
| No | Referent | | Referent | |
| Leash policy prevents pet exposure to rabid animals | | | | |
| Yes | | 0.39 (0.23–0.68) | 0.27 (0.17–0.44) | |
| No | | Referent | Referent | |
| Years lived in Flagstaff | | | | |
| ≥10 | | | | 3.73 (1.77–7.86) |
| <10 | | | | Referent |
| Sex | | | | |
| F | 0.74 (0.56–0.96) | | | |
| M | Referent | | | |
| Characteristics interaction‡ | | | | |
| Age >60 y | | | | |
| Potential for contact with sick domestic animal | | 0.96 (0.56–1.66) | 1.04 (0.60–1.83) | |
| No potential for contact with sick domestic animal | | Referent | Referent | |
| Age ≤60 y | | | | |
| Potential for contact with sick domestic animal | | 2.78 (1.67–4.63) | 2.20 (1.46–3.27) | |
| No potential for contact with sick domestic animal | | Referent | Referent | |
| Women | | | | |
| Aware of quarantine | | 5.42 (3.29–8.95) | | |
| Not aware of quarantine | | Referent | | |
| Men | | | | |
| Aware of quarantine | | 1.37 (0.79–2.39) | | |
| Not aware of quarantine | | Referent | | |

*Characteristics and interactions significant in the multivariate regression analysis, p<0.05.
†Knowledge of rabies in Flagstaff is defined as "yes" if the respondent 1) knew about the outbreak in Flagstaff and 2) knew the 3 main animals that have had rabies in Flagstaff (bat, skunk, fox).
‡Odds ratio estimates for individual terms involved in interaction are not displayed.

these outbreaks have been associated with an increased number of visits to the emergency department of a local hospital, where 48% of persons with animal bite-related visits required rabies PEP in 2009. Heightened vigilance and continued laboratory-based surveillance are warranted in the immediate vicinity and surrounding areas.

Educational efforts were initiated by Coconino County during the current and previous epizootics (*6*). Residents of Flagstaff have received educational messages about rabies and the existing outbreaks through many methods, which likely had a positive effect on the extent of knowledge retained by community members.

Although residents had a general knowledge of rabies as a disease, a large number of persons did not give correct answers to some general knowledge questions, including routes of exposure and animals that can be infected. These misconceptions have been noted in other surveys (*10*,*11*). Future efforts should consider including information about which animals have been reported as rabid in the community and what animals are susceptible. Furthermore, education efforts should focus on specific exposure routes of concern and address possible misconceptions regarding the infectious nature of other bodily fluids such as blood, urine, feces, or skunk spray. This information could play a key role in reducing public concern about rabies virus exposure from noninfectious routes.

Most respondents reported appropriate medical responses to being bitten or scratched by an animal, which include washing of the wound and seeking medical care. Decisions on the risk for rabies and administration of rabies PEP should be made by medical professionals with consultation from local or state public health professionals (*12*). Information about appropriate actions after animal exposure should be maintained in future outreach materials.

The City of Flagstaff Animal Control and Coconino County Public Health Services District Animal Management Office respond to calls related to wild and domestic animals, while the Arizona Game and Fish Department responds only to calls related to wild animals. In contrast

to a large number of respondents (>40%) indicating that they would notify one of the agencies of an ill animal on their property, 32% of persons who had seen an ill animal (including bat, skunk, and fox) did nothing. Regardless of these differences, clear, concise instruction about which agency should be notified would be useful for residents and may help streamline notification.

During the recent rabies outbreak in Flagstaff, human and pet exposures occurred from encounters with rabid foxes and skunks. Rabid animals exhibit aggressive or altered behavior which puts others at risk. However, in some circumstances, human-animal contact is a result of the person initiating contact with the animal. Some respondents indicated that they would put themselves in direct contact with ill or nuisance wild animals, and some have trapped and translocated nuisance animals, primarily skunks. The county provides traps for residents to use, with the request that residents bring trapped animals to animal control. This service increases the likelihood that some residents will 1) come into contact with an unknown animal and 2) may translocate that animal. This analysis identified living in Flagstaff for at least 10 years as a characteristic associated with translocators. Long-term residents may be more aware of traps provided by the county.

Approximately half of respondents who have translocated animals moved the animal to an area >5 miles from their property. Thus, long range movement of reservoirs, possibly outside of the trap-vaccinate-release area, has probably occurred. Consequently, not only does translocation expand the range of an outbreak, but removal of target species could diminish local herd immunity by removal of vaccinated animals. Translocation of animals threatens the success of control programs and the spread of rabies has been attributed to translocation (*13*,*14*). Continued outreach, to the community and nuisance operators, should emphasize the risks of translocation to humans, animal populations, and rabies control programs. No local ordinances address the topic or prohibit translocation in Coconino County. State and local ordinances and enforcement should be considered to prevent translocation of rabies reservoirs.

This study found that pet owners had a basic knowledge of rabies and the quarantine. A recent survey conducted in Texas found that dog owners knew more specific facts about rabies than persons who did not own dogs (*15*). Several respondents in Flagstaff noted learning about the outbreak from their veterinarian. This survey did not assess specifics of veterinarian instruction to pet owners; however, this would be a useful avenue of study (*15*). Dog owners were less likely to believe that a mandatory leash policy would help prevent exposure to rabid animals. Local trails are popular destinations for dog owners, and dogs are frequently taken off the leash on these trails. Outreach about exposures and risks to humans and their pets may be warranted for dog owners in particular.

The households in the quarantine area that participated in this study have a larger number of dogs (0.71 for every household) than the estimated national average of 0.63, and an average number of cats the same as the national average (0.48 per household) (*16*). Whether the high proportion of vaccinated animals found in this survey is a reflection of the demographics of the households or a result of the ongoing outbreak and quarantine regulations, is unknown. Vaccination of dogs, but not cats, is required in Arizona (*1*), and Arizona utilizes the vaccination scheme recommended in the 2008 Compendium of Animal Rabies Prevention and Control which recommends that dogs and cats be vaccinated at 3 months of age, 12 months of age, and a receive a booster every year or every 3 years, depending on vaccine label specifications (*17*). Cats are the leading domestic animal reported with rabies in the United States, and consequently, cats are responsible for a substantial proportion of rabies exposures to persons (*18*). Vaccination of companion animals that have regular human contact is a basic, simple, and critical barrier to exposure. Veterinarians and public health, and animal control personnel should emphasize vaccination of domestic dogs and cats. Continued education and vaccination measures will help alleviate risk to companion animals, and subsequently, to humans.

This study has several limitations. First, only household members in Flagstaff who responded to the survey are characterized in this study, and without characterization of nonresponders, a nonresponse bias cannot be evaluated. Also, not all questions were answered by all respondents. Compared with the 2000 US Census data for Flagstaff (*19*), the survey respondents were older (68% vs. 5.3% ≥65 years of age), more likely to have a college degree (75% vs. 39.4%), and more likely to be female (59% vs. 50.4%). Taken together, these demographics may have biased the study in regards to rabies knowledge, but these differences are not necessarily correlated with increased rabies knowledge. In addition, this survey was paper-based with the option to respond to an online version. Less than 2% of completed surveys were Internet-based. The results may be biased and reflect a population that is more likely to complete a paper-based survey versus using social media or a survey administered through email. Also, data from factors such as language barriers and social economic status were not collected, and the results may be affected by such factors.

The findings of this study provide helpful information for county public health in support of their community outreach efforts and where additional efforts might be focused. In particular, a focus on reinforcing rabies virus transmission routes and exposure guidelines should help

reduce public concern about nonexposure events and possibly reduce inquiries to health authorities about such events. This information will be helpful in the event of a future outbreak in Flagstaff or for reference in surrounding areas, especially if rabies expands outside Flagstaff and Coconino County. Rabies has not been reported on the adjacent Navajo Nation for many years. Additional measures would be necessary to tailor prevention and control activities if rabies was to reemerge in this area. In addition to existing messages distributed by media, local public agencies may wish to bolster their existing internet information for the community, as well as outreach through local veterinarians. Outreach to physicians should also be conducted, to reinforce current Advisory Committee on Immunization Practices recommendations on human rabies prevention and PEP administration, as well as to encourage consultation with local and state public health officials to assist with exposure assessment.

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# *Trichomonas vaginalis* Antimicrobial Drug Resistance in 6 US Cities, STD Surveillance Network, 2009–2010

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Nitroimidazoles (metronidazole and tinidazole) are the only recommended drugs for treating *Trichomonas vaginalis* infection, and previous samples that assessed resistance of such isolates have been limited in geographic scope. We assessed the prevalence of in vitro aerobic metronidazole and tinidazole resistance among *T. vaginalis* isolates from multiple geographic sites in the United States. Swab specimens were obtained from women who underwent routine pelvic examinations at sexually transmitted disease clinics in 6 US cities. Cultured *T. vaginalis* isolates were tested for nitroimidazole resistance (aerobic minimum lethal concentration [MLC] ≥50 µg/mL). Of 538 *T. vaginalis* isolates, 23 (4.3%) exhibited low-level in vitro metronidazole resistance (minimum lethal concentrations 50–100 µg/mL). No isolates exhibited moderate- to high-level metronidazole resistance or tinidazole resistance. Results highlight the possibility that reliance on a single class of antimicrobial drugs for treating *T. vaginalis* infections may heighten vulnerability to emergence of resistance. Thus, novel treatment options are needed.

Trichomoniasis, caused by *Trichomonas vaginalis,* is one of the most common nonviral sexually transmitted diseases (STDs): annually, ≈248 million incident cases occur worldwide, and ≈7.4 million cases occur in the United States (*1*,*2*). The estimated US prevalence of *T. vaginalis* infection is 3.1%, with a higher prevalence among black women and women of low socioeconomic status than among other women (*3*). Trichomoniasis is a frequent cause of vaginitis and can contribute to premature rupture of membranes during pregnancy, preterm birth, low birth weight, and may facilitate HIV acquisition (*4*–*7*).

The Centers for Disease Control and Prevention (CDC) (Atlanta, Georgia, USA) STD Treatment Guidelines recommends the use of a 5-nitroimidazole antimicrobial agent, either metronidazole or tinidazole, for the treatment of *T. vaginalis* infection (*8*). Metronidazole has been the mainstay of treatment for several decades; however, tinidazole has better in vitro activity and is well tolerated (*8*).

The reliance on a single drug class for treating *T. vaginalis* infections may be problematic if resistance to nitroimidazole becomes widespread in *T. vaginalis* strains. Three small studies that examined the prevalence of in vitro resistance in the United States have been conducted during the past 15 years, but they were limited in geographic scope (*9*–*11*). Our objective was to assess the prevalence of in vitro aerobic metronidazole and tinidazole resistance among a broad sample of *T. vaginalis* isolates from multiple geographic sites in the United States.

## Methods

Demographic data and *T. vaginalis* isolates were collected from women attending 6 STD clinics participating in the STD Surveillance Network (SSuN). SSuN is a sentinel site surveillance network which, through the implementation of common protocols for collecting, reporting, and analyzing enhanced surveillance data, aims to improve the capacity of national, state, and local STD programs to detect, monitor, and respond rapidly to trends in STDs. *T. vaginalis* specimens from women undergoing

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physical examinations were systematically collected, either consecutively or on selected days of the week, during 2009 and 2010, in the following cities: Birmingham, Alabama (n = 80 viable isolates submitted); Denver, Colorado (n = 99); New York, New York (n = 93); Philadelphia, Pennsylvania (n = 103); San Francisco, California (n = 85); and Seattle, Washington (n = 96). Two sites (Seattle and San Francisco) restricted participation to symptomatic women. (Because the data were obtained through a surveillance activity, CDC did not to require human subjects review.)

Data regarding patient's demographic characteristics, pregnancy status, presence or absence of symptoms suggestive of trichomoniasis (vaginal discharge, odor, or itching), prior trichomoniasis diagnosis in the preceding 12 months, and presence or absence of vaginal discharge on physical examination were abstracted from medical records. Vaginal secretions were collected by using a sterile Dacron swab during the pelvic examination, and the swab was used to inoculate the InPouch TV (BioMed Diagnostics, San Jose, CA, USA) culture media, according to manufacturer's specifications. Before inoculation, the InPouch TV culture medium was stored at room temperature (18°C–25°C) in a horizontal position away from direct sunlight. Inoculated InPouch TV cultures were incubated at 35°C–37°C for 24–96 hours. A culture was considered positive if at least 1 trichomonad was observed by microscopy. Positive cultures were transported to the Division of Parasitic Diseases and Malaria Laboratory (CDC) by overnight express mail. Upon arrival, parasites were incubated in Diamonds TYM (typticase, yeast extract, and maltose medium) at 37°C until axenic cultures were obtained.

Isolates were assayed for metronidazole and tinidazole susceptibility under aerobic conditions, according to the method developed by Meingassner and Thurne using serial dilutions of drug concentrations from 0.2 to 400 µg/mL (*12*). The minimum lethal concentration (MLC) was the lowest dilution at which no motile trichomonads could be observed from an isolate assay. Isolates were tested in triplicate, and the assay was repeated twice. Control strains were CDC 085 (resistant) and CDC 520 (sensitive). If results differed, the modal result was used. Low-level resistance was defined as aerobic MLC 50–100 µg/mL, moderate-level resistance as 200 µg/mL, and high-level resistance as ≥400 µg/mL (*13*).

If multiple isolates were submitted from a single patient, we included the first submitted isolate in the analytic dataset. We compared median MLCs of metronidazole and tinidazole by using the Wilcoxon matched pairs signed rank test to account for intra-isolate correlation. We assessed the prevalence of metronidazole and tinidazole resistance among isolates and compared the prevalence of resistance to each agent by geographic site by using the $\chi^2$ test. We compared median metronidazole MLCs by geographic site by using the Wilcoxon rank sum test. Demographic and clinical data for women infected with a resistant isolate (metronidazole or tinidazole MLC ≥50 µg/mL) were compared with data for women infected with a susceptible isolate by using $\chi^2$ or Fisher exact test for dichotomous data and *t* test for continuous data. p values were 2-tailed and considered significant at $p<0.05$. Analyses were conducted by using SAS version 9.2 (SAS Institute, Cary, NC, USA).

## Results

From April 6, 2009, through November 17, 2010, a total of 560 evaluable vaginal swab specimens were submitted from 538 women (range per woman, 1–3 swab specimens). Of these women, the median age was 28 years (range 13–67 years); 71% were African-American, 11% were non-Hispanic white, 11% were Hispanic or Latina, and 5% were of other race/ethnicity. Race/ethnicity data were missing for 2%. At least 1 previous episode of trichomoniasis was reported by 39% of women who submitted samples. Three percent of the women were pregnant, and none were HIV infected. Symptoms consistent with trichomoniasis (vaginal discharge, odor, or pruritus) were reported by 77% of women. By site, 80 (14.9%) women were from Birmingham, 94 (17.4%) were from Denver, 92 (17.1%) were from New York, 103 (19.1%) were from Philadelphia, 82 (15.2%) were from San Francisco, and 87 (16.2%) were from Seattle.

Of 538 isolates, the median MLC of metronidazole (3.1 µg/mL) was higher than the median MLC of tinidazole (0.8 µg/mL) ($p<0.001$) (Figure). The prevalence of low-level metronidazole resistance was 4.3% (95% CI, 2.7%–6.4%). No isolates exhibited moderate- to high-level metronidazole resistance; all isolates were susceptible to tinidazole. The prevalence of metronidazole resistance did not vary significantly by geographic site: Birmingham, 1.3% (95% CI, 0.1%–6.8%); Denver, 7.5% (95% CI, 3.1%–14.7%); New York, 2.2% (95% CI, 0.3%–7.6%); Philadelphia, 3.9% (1.1%–9.7%); San Francisco, 4.9% (95% CI, 1.3%–12.0%); and Seattle, 5.8% (95% CI, 1.9%–12.9%). The median metronidazole MLC among isolates from Birmingham (3.1 µg/mL [range 0.4–50 µg/mL]) was lower than among isolates from Seattle (6.3 µg/mL [range 0.4–100 µg/mL]; p = 0.043); otherwise no significant differences in median MLC by site were detected. We did not find significant differences between women infected with a metronidazole-resistant strain and women infected with a metronidazole-susceptible strain in terms of age, race/ethnicity, pregnancy status, symptom status, or having a previous diagnosis of trichomoniasis.

Sixteen women submitted 2 evaluable isolates, and 1 woman submitted 3 isolates. Among these 17 women, the median duration between sample collection was 128 days

(range 2–392 days). Three women submitted the second specimens within 30 days of the initial submission (initial MLCs of metronidazole: 0.8–3.1 µg/mL), 3 within 60 days (initial MLCs 1.6–6.3 µg/mL), and 1 within 90 days (initial MLC 0.8 µg/mL). Data on sexual re-exposure were available for only 1 of these 7 women. For initial isolates from 2 women, MLCs of metronidazole were >12.5 µg/mL (50 µg/mL in both cases). In 1 case, the second isolate was collected 158 days after the first, and the woman reported 12 sex partners in the preceding 3 months. In the other case, the second isolate was collected 308 days later, and the MLC of metronidazole for this strain was 0.8 µg/mL.

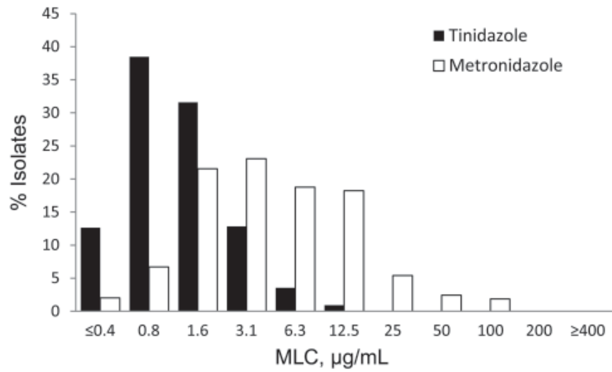


Figure. Distribution of minimum lethal concentrations (MLCs) of tinidazole and metronidazole, STD Surveillance Network, 2009–2010 (n = 538). Susceptibility to metronidazole and tinidazole are defined as MLC ≤25 µg/mL, low-level resistance as MLC 50–100 µg/mL, moderate-level resistance as MLC 200 µg/mL, and high-level resistance as MLC ≥400 µg/mL.

## Discussion

To our knowledge, this study is the first multisite evaluation conducted to assess the prevalence of in vitro *T. vaginalis* resistance in the United States. Although metronidazole has been used to treat *T. vaginalis* infections for ≈40 years, we found a low prevalence of in vitro metronidazole resistance. MLCs of tinidazole were lower than MLCs of metronidazole, and we did not detect tinidazole resistance.

The prevalence of in vitro metronidazole and tinidazole resistance is consistent with previously published US estimates. Three studies conducted in the southeastern United States among women attending STD or gynecology clinics from 1997 through 2005 found a metronidazole-resistance prevalence of 2.4%–9.5% (*9–11*). Most metronidazole-resistant isolates in these studies exhibited low-level resistance. In 2 of these studies tinidazole resistance also was tested: Krashin et al. did not detect tinidazole resistance (*11*), and Schwebke and Barrientes detected 1 isolate (0.6%) that exhibited low-level tinidazole resistance (MLC 50 µg/mL) among the 178 isolates tested (*10*). Among 91 isolates collected in Spain during 1995 and 1999, 2.2% exhibited low-level resistance to metronidazole (*14*). A small study conducted among women from Papua New Guinea found 21 (91%) of 23 studied isolates had MLCs of metronidazole of ≥50 µg/mL, including 4 (17%) with MLCs of 200 µg/mL (*15*). However, the sampling method used to enroll women was not described and may not have been systematic, thus substantially limiting the ability to estimate the population-level prevalence of resistance. Investigators in the United Kingdom reported that 1.7% of women treated for trichomoniasis during 1998–2002 appeared to have not responded to treatment and denied re-exposure; in vitro susceptibility data were not available (*16*).

Inconsistency does exist between in vitro susceptibility results and clinical outcomes of treatment, particularly for infections with low-level in vitro resistance. Clinical resistance and treatment failure have occurred with *T. vaginalis* isolates for which MLCs of nitroimidazoles were as low as 12.5 µg/mL, and treatment success has occurred in infections with *T. vaginalis* isolates for which MLCs of nitroimidazoles were 100–200 µg/mL (*13*). In general, however, elevated MLCs are associated with a greater likelihood of treatment failure. A recent evaluation of the utility of susceptibility testing in women for whom clinical treatment has failed found that treatment recommendations based on susceptibility results may have a beneficial role in informing the clinical management of some women with persistent infection (*17*). *T. vaginalis* susceptibility testing is not available routinely; such testing should be conducted by a qualified laboratory and is available at CDC (1–800-CDC-INFO).

As a cross-sectional evaluation of in vitro antimicrobial drug susceptibility, the study was not designed to detect clinical treatment failures. Multiple isolates were collected from 17 women. However, we did not systematically collect data on sexual re-exposure after treatment or adherence, so we were not able to determine whether any of these cases resulted from treatment failure. The MLCs of metronidazole for the initial isolates were low, suggesting that clinical resistance was unlikely. In 2 cases, the initial isolate exhibited low-level resistance (MLC of metronidazole 50 µg/mL) and a second isolate was later collected. Both of these cases were probably re-infections.

For isolates in our study, MLCs of tinidazole were lower than those of metronidazole, which supports the idea that tinidazole should be prescribed for patients whose infections do not respond clinically to metronidazole. This finding is consistent with results of previous studies which showed that tinidazole had better in vitro activity than metronidazole at similar molar concentrations (*18*). Tinidazole has a longer serum half-life than metronidazole

and exhibits good tissue penetration (*19*), yet is more expensive than metronidazole. Although tinidazole and metronidazole are the only nitroimidazoles available in the United States, ornidazole, tenonitrazole, and nimorazole are available in Europe and could be alternatives to metronidazole. These agents are of the same drug class as metronidazole, however, and the emergence of clinically notable nitroimidazole resistance would be expected to adversely influence the treatment effectiveness of each of these agents.

This study had several limitations. First, the sample was limited to women attending STD clinics participating in SSuN; thus, our findings are not representative of the general population. In addition, symptomatic women were likely to have been overrepresented because participating women were seeking care in STD clinics, and 2 of the sites sampled only symptomatic women. Also, although we believe this is the largest study of its kind, the sample size may not have been large enough to detect significant differences across sites, nor to detect tinidazole resistance or high-level metronidazole resistance. That we did not detect isolates with such resistance suggests that its prevalence in this population is low. CDC occasionally receives isolates that are highly resistant to metronidazole or tinidazole, however (W.E. Secor, pers. comm.).

Although the prevalence of resistance is currently low in the United States, reliance on a single class of antimicrobial drugs heightens vulnerability if clinical *T. vaginalis* nitroimidazole resistance becomes widespread. Market forces alone are unlikely to spur the development of new anti-trichomonal drugs. Further evaluation of existing compounds and development of novel systemic treatment options are needed, and efforts to promote and support antimicrobial drug development and evaluation are warranted.

In summary, we found a 4% prevalence of low-level metronidazole resistance among *T. vaginalis* isolates from women attending several STD clinics throughout the United States. Periodic sentinel surveillance evaluations of *T. vaginalis* antimicrobial drug susceptibility should be carried out to monitor the possible emergence of resistance.


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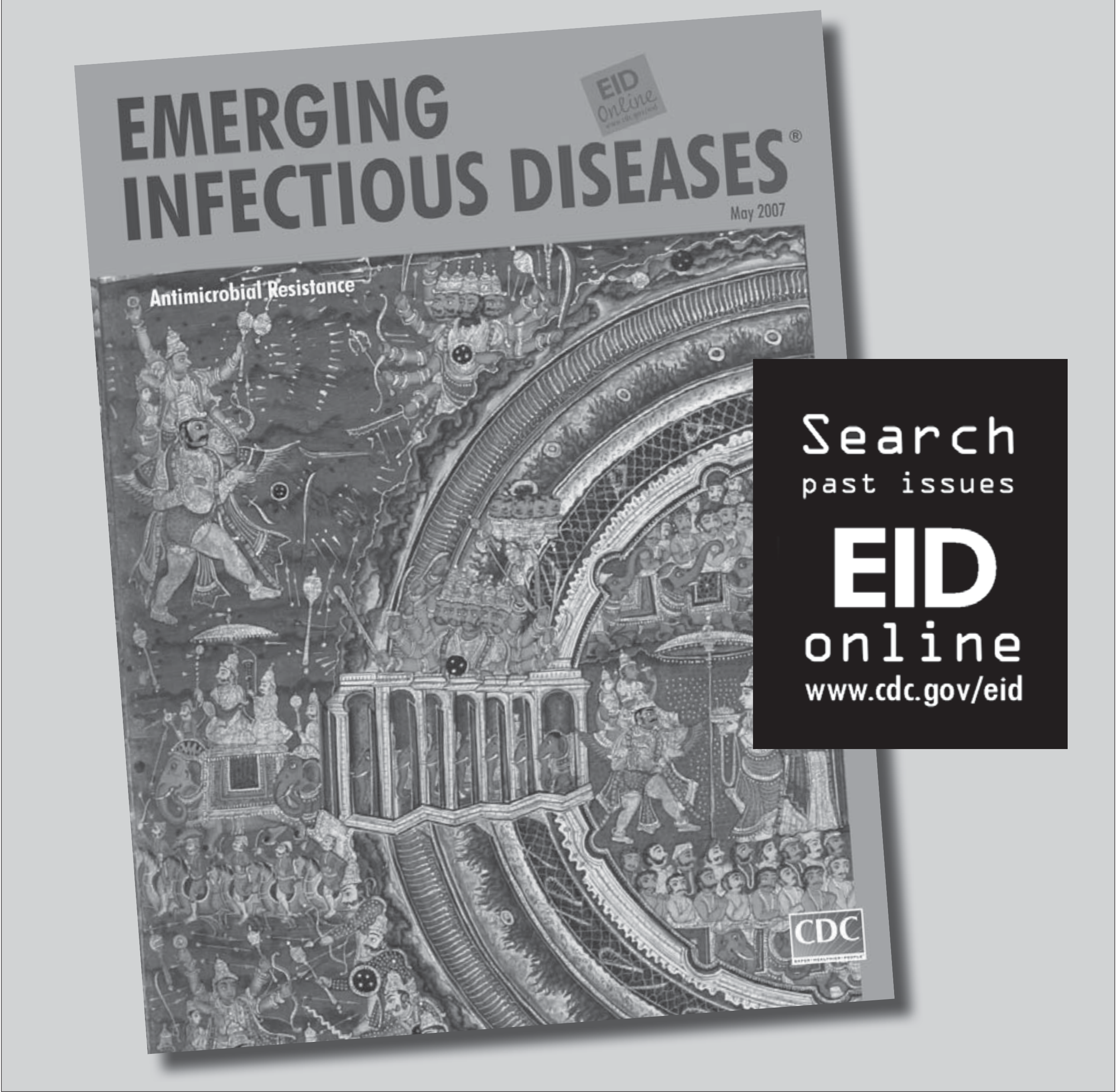

# Virulence Potential of Fusogenic Orthoreoviruses

**Ann H. Wong, Peter K.C. Cheng, Mary Y.Y. Lai, Peter C.K. Leung, Kitty K.Y. Wong, W.Y. Lee, and Wilina W.L. Lim**

Several severe respiratory virus infections that have emerged during the past decade originated in animals, including bats. In Indonesia, exposure to bats has been associated with increased risk of acquiring orthoreovirus infection. Although orthoreovirus infections are mild and self-limiting, we explored their potential for evolution into a more virulent form. We used conventional virus culture, electron microscopy, and molecular sequencing to isolate and identify orthoreoviruses from 3 patients in whom respiratory tract infection developed after travel to Indonesia. Virus characterization by plaque-reduction neutralization testing showed antigenic similarity, but sequencing of the small segment genes suggested virus reassortment, which could lead to increased virulence. Bats as a reservoir might contribute to virus evolution and genetic diversity, giving orthoreoviruses the potential to become more virulent. Evolution of this virus should be closely monitored so that prevention and control measures can be taken should it become more virulent.

The virus family *Reoviridae* comprises a diverse group of nonenveloped, segmented, double-stranded RNA viruses classified into 10 genera. Members of the genus *Orthoreovirus* are classified as fusogenic or nonfusogenic, depending on their ability to cause syncytium formation in cell culture. Genetically, orthoreoviruses contain 10 segments: 3 large, 3 medium, and 4 small (S1, S2, S3, and S4). The characteristic of the segmented genome facilitates virus reassortment, which contributes to virus diversity. Sequencing studies indicate that reassortment in nature is a major determinant of reovirus evolution (*1*,*2*).

In 2007, Chua et al. reported the isolation of Melaka virus, a novel fusogenic reovirus of bat origin, which has been associated with acute respiratory illness in humans (*3*). Subsequently, other related strains of bat-associated orthoreoviruses have been reported, including Kampar virus from Malaysia and Xi River virus from the People's Republic of China (*4*,*5*). The role of bats as reservoirs of various zoonotic viruses, such as Nipah, Hendra, coronaviruses, and lyssavirus, has brought attention to the role of bats in virus evolution and emerging infections in humans (*6*–*10*).

We isolated and characterized 3 fusogenic orthoreoviruses from 3 travelers who had returned from Indonesia to Hong Kong during 2007–2010. Using a plaque-reduction neutralization test (PRNT), we confirmed infection in 2 of these patients and showed cross-reactivity of antibodies among the 3 virus strains. To track virus evolution and provide evidence of genetic reassortment, we used PCR sequencing and phylogenetic analysis to compare their genetic relatedness.

## Methods

### Case Descriptions

Patient 1 was a 51-year-old male tourist from Bali, Indonesia, who became ill in April 2007. This case has been reported elsewhere (*11*). The patient left Hong Kong before a serum sample could be obtained.

Patient 2 was a 26-year-old woman who sought care at a designated fever clinic during the outbreak of pandemic influenza in July 2009. She had a 2-day history of influenza-like illness (ILI), and oseltamivir was prescribed. Throat and nasal swab specimens were taken 3 days after onset of illness. Serum was collected 59 days after symptom onset. The patient recovered uneventfully. She reported having traveled to Bali, Indonesia, for 5 days and noticing ILI symptoms on the last day of her visit. While in Indonesia, she visited a safari park and entered bat caves on the second day. Her travel companions remained asymptomatic.

Patient 3 was a 29-year-old woman who sought care for ILI at the General Outpatient Clinic in Hong Kong in June 2010 after a recent visit to Indonesia. As part of sentinel

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surveillance for ILI, throat and nasal swab specimens were taken for routine isolation of respiratory viruses (*12*). Serum was collected 8 days after symptom onset.

### Virus Isolation

As part of routine surveillance to monitor influenza activity, throat and nasal swab specimens were inoculated into cells. The cell lines used for culture were MDCK, rhabdomyosarcoma, human laryngeal carcinoma, and rhesus monkey kidney (*12*).

### Virus Identification and Characterization

#### Immunofluorescence Testing

For samples that exhibited syncytial cytopathic effect (CPE) on cell culture, we used immunofluorescence testing with monoclonal antibodies to preliminarily identify viruses (*12*). We tested for viruses commonly associated with syncytial CPE formation on cell culture: respiratory syncytial virus, measles virus, and mumps virus. As part of routine investigation to exclude infection with influenza A(H1N1)pdm09 virus during the time of the pandemic influenza outbreak, a sample from patient 2 was also tested for influenza A.

#### Electron Microscopy

For viruses that had negative immunofluorescence results for respiratory syncytial, mumps, and measles virus monoclonal antibodies, we processed supernatant of tissue culture fluid for examination of virus morphologic appearance by electron microscopy. For negative staining, 1 drop of culture supernatant was adsorbed on Formvar carbon coated grid (1 min), stained with 3% phosphotungstic acid (pH 6.3) (1 min), and inactivated with ultraviolet irradiation before examination at 80 KV with a Philips transmission electron microscope (Eindhoven, the Netherlands).

#### PCR, Nucleotide Sequencing, and Phylogenetic Analysis

To further identify the virus and its phylogeny, we next designed primers based on published sequences of Melaka virus, selective for the S1 to S4 segments of orthoreovirus (*3*). In brief, by using a QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) according to manufacturer's instructions, we extracted viral RNA from 200 μL of culture supernatant of the 3 orthoreoviruses. Virus segments S1–S4 were amplified by using a QIAGEN One-Step RT-PCR Kit with primers shown in the Table. Reverse transcription PCR was performed as described (*3*). The amplified products were analyzed by 2% agarose gel electrophoresis. Sanger sequencing was conducted by using an ABI Prism 3130XL DNA Sequencer (Applied Biosystems, Foster City, CA, USA) and an ABI Prism Big Dye Terminator Cycle Sequencing Kit, version 3.1 (Applied Biosystems). Sequence alignment by using Simmonic and phylogenetic analysis of the partial sequences of segments S1 (1,501 nt), S2 (1,213 nt), S3 (1,078 nt), and S4 (1,113 nt) was performed by using MEGA4 software (www.megasoftware.net) with the neighbor-joining method. Sequences were submitted to GenBank and compared with other orthoreovirus sequences for genetic relatedness and evolution (Figures 1–4).

Table. Primers used to amplify the 4 small gene segments of fusogenic orthoreovirus from 3 travelers who had returned from Indonesia to Hong Kong during 2007–2010

| Sequence, 5′ → 3′ | Polarity |
|---|---|
| Segment 1 | |
| ATGAGTGGTGATTGTGC | Forward |
| GATCTGATCGCGAAGCG | Reverse |
| GCTTTGATCATGACGATG | Forward |
| GTGTCGATTGAGAAAGTGA | Reverse |
| Segment 2 | |
| CCCACGGACCAACGACAAC | Forward |
| AAAGCGCTTAGCTGAGAAGCG | Reverse |
| GAGACGTGGCCTAATATGTTG | Forward |
| GATGATTAGACCACGGCTGAG | Reverse |
| Segment 3 | |
| GCGGGTACTGGGTCTCAGA | Forward |
| GCTTAGCACCAAAGGAAGTTACG | Reverse |
| GTCATCGCGAAGTTTTCAGAAC | Forward |
| CTCCTGTCGATGCTCACCA | Reverse |
| Segment 4 | |
| CGCAAGCCCAGATGGAGGT | Forward |
| CGGAGTGACAATGACATGTTCAG | Reverse |
| CGTTTCCACATCATCGCTGTC | Forward |
| AGAGCATAGTGCGATGGTGTC | Reverse |

#### PRNT

For PRNT, we first prepared a 1:5 diluted serum sample by using 2% minimal essential medium (MEM) and heat inactivation at 56°C for 30 min. We then performed serial

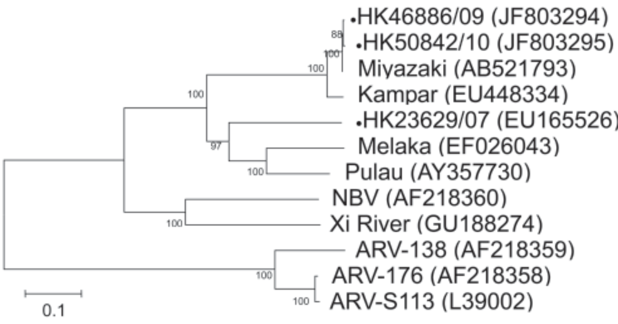


Figure 1. Phylogenetic tree of orthoreoviruses based on partial sequence alignment of the cell attachment protein (S1 gene segment). GenBank accession number for each sequence is in parentheses after the virus name. Numbers at nodes indicate bootstrap values based on 1,000 replicates. Dots indicate viruses isolated from 3 travelers who had returned from Indonesia to Hong Kong during 2007–2010. Scale bar indicates nucleotide substitutions per site. ARV, avian reovirus; NBV, Nelson Bay virus.

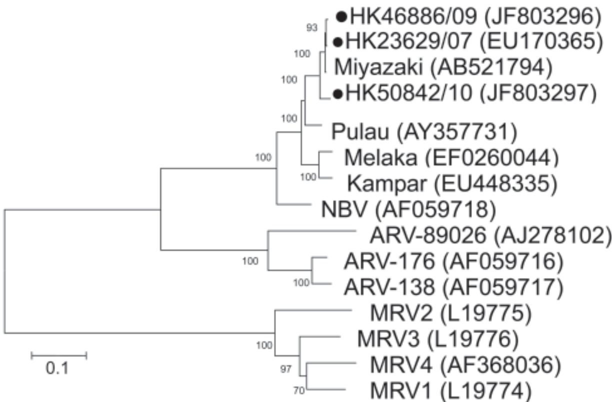


Figure 2. Phylogenetic tree of orthoreoviruses based on partial sequence alignment of the major inner capsid protein (S2 gene segment). GenBank accession number for each sequence is in parentheses after the virus name. Numbers at nodes indicate bootstrap values based on 1,000 replicates. Dots indicate viruses isolated from 3 travelers who had returned from Indonesia to Hong Kong during 2007–2010. Scale bar indicates nucleotide substitutions per site. ARV, avian reovirus; MRV, mammalian reovirus; NBV, Nelson Bay virus.

double dilutions of serum samples from 1:5 to 1:2,560. Equal volumes of serum and virus mixture were incubated at 37°C for 2 h. Then 200 μL of serum and virus mixture with final dilutions of 1:10 to 1:5,120 was added to each of the 6-well plates containing a monolayer of confluent Vero cells ($2 \times 10^5$ cells/mL in 2% MEM). Serum, virus, and cell controls were included in the assay. After 1 h of virus absorption, the inoculum was discarded, plates were washed with 2% MEM, 4 mL of overlay LE agar was added, and the plates were incubated at 37°C in a $CO_2$ incubator for 4 days. The end-point neutralizing antibody titer was determined by serum with the highest dilution that reduced plaques by 70% more than the virus control (*12*).

## Results

### Virus Identification

All 3 orthoreoviruses (designated HK23629/07, HK46886/09, and HK50842/10) showed syncytial CPE in culture tubes after 3 to 4 days of incubation in each of the 4 cell lines. (Orthoreoviruses also cause CPE in MRC5 [human diploid lung] and Vero cells.) Immunofluorescent testing indicated that the samples were negative for influenza, respiratory syncytial, measles, and mumps viruses. Electron microscopy revealed virus morphologic appearance consistent with that of a reovirus (Figure 5).

### Neutralizing Antibody Titers

According to PRNT results, serum of patient 2 had a neutralizing antibody titer of 1,280 against HK46886/09 virus and HK23629/07 virus and a titer of 640 against HK50842/10 virus. Serum of patient 3 had a neutralizing antibody titer of 20 against all 3 viruses. The control serum had a neutralizing antibody titer <10.

### Nucleotide Sequences and Phylogeny

For the S1 virus cell attachment protein, the HK46886/09 and HK50842/10 viruses were clustered with the Miyazaki virus and related closely to the Kampar virus isolated in Malaysia; the S1 virus cell attachment protein of the HK23629/07 virus has been reported as similar but distinct from Melaka virus (*11*) (Figure 1). For the S2 major inner capsid protein and the S4 major outer capsid protein, all 3 viruses were clustered together and closely related to Miyazaki virus (Figures 2, 4). For the S3 nonstructural protein, the HK46886/09 and HK50842/10 viruses were clustered together and similar to Kampar and Paula viruses, whereas the HK23629/07 virus was clustered with Miyazaki virus (Figure 3). Sequence analysis of the S1–S4 segments showed that HK46886/09 and HK50842/10 viruses were similar strains and different from the HK23629/07 virus. The difference in sequence homology between segments of the virus strains suggested reassortment events.

## Discussion

The isolation and epidemiologic studies of Melaka and Kampar virus in Malaysia provide evidence that a novel group of fusogenic orthoreovirus of bat origin is associated with human respiratory tract infection. The role of bats as reservoirs and sources of potential emerging viral infections has been extensively studied with regard to Nipah, Hendra, lyssavirus, and coronaviruses (*6*,*7*,*10*). Although we were unable to demonstrate rises in antibody titers because of a lack of paired acute-phase and convalescent-phase serum samples, we believe that the isolation of fusogenic orthoreoviruses, the presence

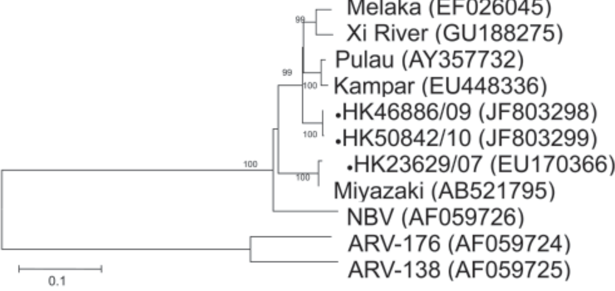


Figure 3. Phylogenetic tree of orthoreoviruses based on partial sequence alignment of the nonstructural protein (S3 gene segment). GenBank accession number for each sequence is in parentheses after the virus name. Numbers at nodes indicate bootstrap values based on 1,000 replicates. Dots indicate viruses isolated from 3 travelers who had returned from Indonesia to Hong Kong during 2007–2010. Scale bar indicates nucleotide substitutions per site. ARV, avian reovirus; NBV, Nelson Bay virus.

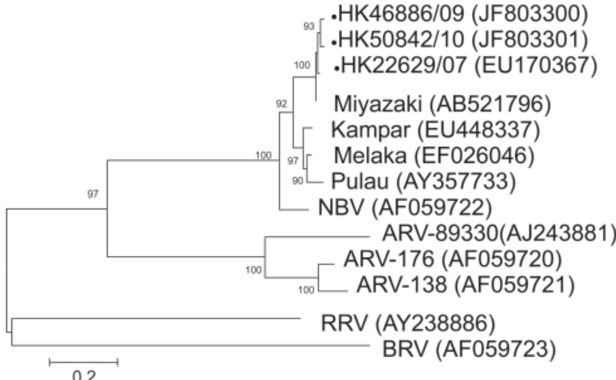


Figure 4. Phylogenetic tree of orthoreoviruses based on partial sequence alignment of the major outer capsid protein (S4 gene segment). GenBank accession number for each sequence is in parentheses after the virus name. Numbers at nodes indicate bootstrap values based on 1,000 replicates. Dots indicate viruses isolated from 3 travelers who had returned from Indonesia to Hong Kong during 2007–2010. Scale bar indicates nucleotide substitutions per site. ARV, avian reovirus; BRV, baboon reovirus; NBV, Nelson Bay virus; RRV, reptilian reovirus.

of antibody to the viruses, and epidemiologic information suggest that orthoreoviruses were possibly associated with the patients' upper respiratory tract infections.

Similar to infection caused by Melaka virus, the infection in the patients reported here was a mild upper respiratory infection followed by full recovery. Also, similar to the 7-day incubation period of the patient infected with Melaka virus, incubation periods for the patients reported here ranged from 4 to 7 days. The cross-reactivity of the 3 virus strains, demonstrated by PRNT, suggests that they were antigenically similar. Neutralizing antibodies appeared within 8 days of disease onset, and titers were still high at 2 months. Although all persons who had been in contact with the patients during their travel and at home were asymptomatic, no serum was available for further workup to determine subclinical infection or human-to-human transmission. Nevertheless, subclinical infection and transmission among close contacts have been demonstrated (*3*,*4*).

Phylogenetic analysis of the partial S1–S4 gene segments demonstrated genetic diversity of the viruses. Although the S2 major inner capsid proteins and S4 major outer capsid proteins of all 3 viruses were clustered together, they differed in the S1 virus cell attachment protein and S3 nonstructural protein. The S1 segments of HK46886/09 and HK50842/10 viruses were clustered together with the Miyazaki virus and related to the Kampar virus, but that of the HK23629/07 virus was similar to but distinct from the Melaka virus. The S3 segments of HK46886/09 and HK50842/10 viruses were clustered together and similar to those of the Kampar and Paula viruses, whereas the HK23629/07 virus shared similar homology with the Miyazaki virus. The difference in clustering based on the S1–S4 segments alone provides evidence for virus reassortment.

Epidemiologic information indicated that all 3 orthoreoviruses isolated were related to travel to Indonesia. Isolation of closely related orthoreoviruses within a short time in the region illustrates the genetic diversity of the virus and that reassortment events are not uncommon. The association of orthoreovirus infection with bat exposure showed that these abundant mammals are natural reservoirs and play a contributing role in virus evolution. In addition, the segmented nature of the virus genome also facilitates virus reassortment, which leads to virus diversity.

Outbreaks of emerging infections in the past decade, such as those caused by avian influenza (H5N1) virus, severe acute respiratory syndrome coronavirus, Nipah virus, and Hendra virus, have been associated with animal origins. Infections occurred in humans when the pathogens crossed the species barrier to expand their host range. Changes in human activities also bring humans in close contact with animals (*6*,*8*,*13*–*15*). Epidemiologic and molecular studies provide evidence that the pathogens isolated from humans shared a high degree of homology with viruses isolated from their animal reservoirs (*8*,*15*). The ability of a pathogen to emerge and cause outbreaks depends on the efficiency of its human-to-human spread. Recombination and reassortment events facilitate virus adaptation in the new host. Further study is warranted to unravel the possible virus spillover and adaptation of orthoreoviruses as a cause of human infection.

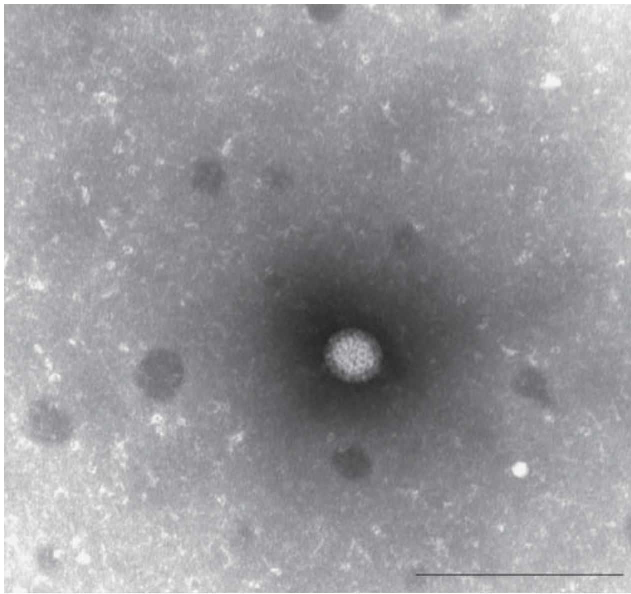

Figure 5. Electron micrograph of orthoreovirus HK50842/10. Scale bar = 200 nm.

Although symptoms of orthoreovirus infection are mild and self-limiting, continued close monitoring of virus evolution and further study are essential. Changes in virulence would signal the need for prevention and control strategies.


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Dr A.H. Wong is a medical virologist at the Public Health Laboratory, Centre for Health Protection, Hong Kong. Her research interests include diagnosis and laboratory surveillance of viral diseases.

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# Intrafamilial Circulation of *Tropheryma whipplei*, France

**Florence Fenollar, Alpha K. Keita, Sylvain Buffet, and Didier Raoult**

*Tropheryma whipplei,* which causes Whipple disease, has been detected in 4% of fecal samples from the general adult population of France. To identify *T. whipplei* within families, we conducted serologic and molecular studies, including genotyping, on saliva, feces, and serum from 74 relatives of 13 patients with classic Whipple disease, 5 with localized chronic *T. whipplei* infection, and 3 carriers. Seroprevalence was determined by Western blot and compared with 300 persons from the general population. We detected *T. whipplei* in 24 (38%) of 64 fecal samples and 7 (10%) of 70 saliva samples from relatives but found no difference between persons related by genetics and marriage. The same circulating genotype occurred significantly more often in families than in other persons. Seroprevalence was higher among relatives (23 [77%] of 30) than in the general population (143 [48%] of 300). The high prevalence of *T. whipplei* within families suggests intrafamilial circulation.

Whipple disease, a rare sporadic disease, was first considered a metabolic disease (*1*) and later suspected to be an infectious disease caused by a rare bacterium, *Tropheryma whipplei* (*2*). However, the causative bacterium is common (*3–5*), and the well-known and classic form of Whipple disease (characterized by periodic acid–Schiff-stained bacilli in infected small-bowel macrophages) represents only 1 rare clinical form of infection caused by *T. whipplei* (*6,7*). In the absence of intestinal lesions, *T. whipplei* is involved in subacute or chronic infections, such as endocarditis (*8*), encephalitis (*2*), uveitis (*9*,*10*), adenopathy (*2*), and osteoarticular infections (*2*,*11*). Recently, *T. whipplei* was reported to cause acute infections, such as pneumonia (*12*,*13*), gastroenteritis (*14*,*15*), and bacteremia (*16*). Asymptomatic carriers have been identified for whom *T. whipplei* prevalence varied widely by geography or occupation (*17–19*). In Europe, the prevalence of *T. whipplei* in fecal samples from the general healthy adult population is ≈1%–11% (*2*,*3*). *T. whipplei* has been detected in sewage and is more prevalent in fecal samples of sewer workers (12%–26%) than in the general population (4%) (*20*,*21*). In a study in 2 rural Senegalese villages, 44% of children 2–10 years of age carried *T. whipplei* in their feces (*4*).

*T. whipplei* genotyping has shown high genetic diversity unrelated to pathogenicity, but this diversity varies geographically between Europe and Africa (*4*,*22*). Some clones circulate in particular communities, suggesting interhuman transmissibility (*4*,*14*). Moreover, the chronic carriage of *T. whipplei* in saliva and feces suggests that the bacterium might be transmissible within the same family. This question was raised to one of us (D.R.) by a person who had chronic carriage of *T. whipplei* in his saliva (*20*) and was concerned about his family. The development of *T. whipplei* serologic assays has enabled delineation between patients with Whipple disease who lack or have weak immune responses against *T. whipplei* and asymptomatic carriers who show strong immune response to the bacterium (*23–25*).

To identify *T. whipplei* within families, during 2003–2011 we conducted molecular and serologic investigations on samples from the families of patients who had chronic *T. whipplei* infection and were asymptomatic carriers. We also studied *T. whipplei* seroprevalence in the population of France, which enabled us to compare the prevalence with that of the families.

## Patients, Materials, and Methods

### Study Participants

The study comprised 18 patients with *T. whipplei* infections and 3 asymptomatic carriers of *T. whipplei*. Our laboratory in Marseille, France, had previously diagnosed

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all the infections, and one of us (D.R.) had followed-up all the patients. Samples from patients were submitted for diagnostic purposes (*3*); samples from carriers were submitted for epidemiologic studies (*20*). Our criteria to confirm classic Whipple disease included presence of positive results by periodic acid–Schiff staining and/or specific immunohistochemical results from small-bowel biopsy specimens. In contrast, the hallmark of localized extra-intestinal infection from *T. whipplei* was absence of these typical histologic lesions (*3*). The criteria for establishing the status of carriers were lack of clinical manifestations and presence of *T. whipplei* DNA within feces or saliva (*20*).

A total of 74 family members of 13 patients (10 men) with classic Whipple disease (26–78 years of age; mean ± SD age 51.4 ± 16 years), 5 (3 women) with localized chronic *T. whipplei* infection (36–71 years of age; mean ± SD age 47.2 ± 15.7 years), and 3 (all men) chronic asymptomatic carriers (27–43 years of age; mean age 33.7 years) participated. Of the family members, 40 were female relatives. Ages of family members ranged from 2 months to 79 years (mean ± SD 38 ± 22 years). A total of 64 fecal and 70 saliva specimens were analyzed by using *T. whipplei*–specific PCR. Serum samples from 30 family members were analyzed by using *T. whipplei* serologic analysis. All patients and their families provided informed consent; parents or guardians provided consent for young children.

For the seroprevalence study, 200 serum samples from blood donors from the French National Blood Service (105 men; ages of all patients 18–65 years [mean ± SD 40.74 ± 12 years]) were analyzed by Western blot. In addition, 100 control serum samples from patients hospitalized in the University Hospitals in Marseille (55 male patients; age range of all patients 1 month–88 years [mean 55.5 years]) were also analyzed by Western blot; these samples were not taken for explicit use in this study.

The overall study was approved by the local ethics committee: Institut Fédératif de Recherche 48, Marseille (agreement no. 09–018). Data obtained from adult *T. whipplei* carriers by using the same techniques were also included for prevalence comparisons (*3*,*20*).

### Molecular Assays

For each patient for whom samples were available, ≈1 g of feces and 200 μL of saliva were submitted for DNA extraction by using the QIAamp DNA MiniKit (QIAGEN, Hilden, German), according to the manufacturer's recommendations. Quantitative real-time PCR was performed by using a LightCycler instrument (Roche Diagnostics, Meylan, France) with the QuantiTect Probe PCR Kit as described by the manufacturer (*3*). Specimens were tested by using the Twhi3F (5′-TTGTGTATTTGGTATTAGATGAAACAG-3′) and Twhi3R (5′-CCCTACAATATGAAACAGCCTTTG-3′) primer pair and the specific TaqMan probe Twhi3 (6-FAM-5′-GGGATAGAGCAGGAGGTGTCTGTCTGG-3′-TAMRA). If a specimen tested positive in this assay, the result was confirmed by a second quantitative PCR by using the Twhi2F (5′-TGAGGATGTATCTGTGTATGGGACA-3′) and Twhi2R (5′-TCCTGTTACAAGCAGTACAAAACA AA-3′) primer set and the Twhi2 probe (6-FAM-5′-GAGAGATGGGGTGCAGGACAGGG-3′-TAMRA).

*T. whipplei* detected in the specimens was genotyped by using multispacer typing as described (*22*). Each of the 4 highly variable genomic sequences from each specimen was compared with the sequences available in GenBank and in our internal laboratory database to determine their corresponding genotype.

### Western Blot

Serologic assays were performed by using Western blot. The native and deglyclosylated *T. whipplei* extracts were prepared, resolved by using sodium dodecylsulfate–polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes as described (*23*,*24*,*26*). The membranes were immersed at room temperature in phosphate-buffered saline supplemented with 0.2% Tween 20 and 5% nonfat dry milk (blocking buffer) for 1 h before incubation with primary serum (diluted 1:1,000 in blocking buffer) for 1 h. The membranes were washed 3× with phosphate-buffered saline–Tween 20. Immunoreactive spots were detected by incubating membranes for 1 h at room temperature with peroxidase-conjugated goat anti-human antibodies (Southern Biotech, Birmingham, AL, USA) diluted 1:1,000 in blocking buffer. The assay was performed to determine the presence of *T. whipplei*–specific IgG in the serum (Southern Biotech). Detection was performed as described (*23*,*24*,*26*). Interpretation was based in particular on the analysis of a *T. whipplei* glycoprotein of 110 kDa, that is a member of the Wnt1-inducible signaling pathway proteins, a family of *T. whipplei*–specific membrane proteins as reported (*23*,*24*,*26*).

### Statistical Analysis

Statistical analyses were performed by using Fisher exact test with Epi Info 6 (www.cdc.gov/epiinfo/Epi6/EI6dnjp.htm). Results were considered statistically significant at $p<0.05$.

## Results

### Molecular Analysis of Saliva and Feces from Family Members

For the 74 family members of *T. whipplei*–infected patients or chronic carriers, the following familial relationships were examined: 12 sons, 10 wives, 8

daughters, 8 mothers, 6 fathers, 4 nephews, 9 grandchildren, 5 sisters, 4 husbands, 3 daughters-in-law, 2 brothers, 1 aunt, 1 stepsister, and 1 stepbrother. Family members who were positive for *T. whipplei* were tested 0–60 months (mean ± SD 33.5 ± 20 months) after treatment of their respective family member for *T. whipplei* infection.

Overall, *T. whipplei* DNA was detected in 24 (38%) of 64 fecal specimens and 7 (10%) of 70 saliva samples (Table 1). The prevalence of *T. whipplei* in feces of family members was significantly higher than that in the general population (4 [4%] of 102, p<0.001) (*20*). *T. whipplei* prevalence was also significantly higher than in feces from patients without Whipple disease (7 [2%] of 299, p<0.001) (*3*) and sewer workers (19 [9%] of 211; p<0.001) (*20*). In addition, the prevalence of *T. whipplei* in saliva from family members was significantly higher than that from patients without Whipple disease (1 [0.3%] of 432; p<0.001) (*3*). Among families of patients with classic Whipple disease, the prevalence of *T. whipplei* in feces was 14 (31%) of 45; for saliva samples, the prevalence was 4 (8%) of 48. For family members of patients with localized *T. whipplei* infection, bacterial prevalence was 2 of 9 in feces and 1 of 12 in saliva samples. Among the families of chronic carriers, *T. whipplei* prevalence was 8 of 10 in feces and 2 of 10 in saliva. For family members of chronic carriers, the prevalence in feces was significantly higher than in any other tested population, including family members of patients who had active *T. whipplei* infections (16 [30%] of 54; p = 0.004).

Of the 16 persons related by marriage, 5 (31%) were positive for *T. whipplei*; of the 54 persons related by genetics, 19 (35%) were positive (p = 0.99). At the time of the study, 26 (35%) of the 74 relatives lived in the same household as the related index patient, and 48 (65%) lived elsewhere. Of the 25 relatives positive for *T. whipplei*, 9 (36%) lived in the same household as the related index patient, and 16 (64%) lived elsewhere. Of the 49 relatives negative for *T. whipplei*, 17 (35%) lived in the same household as the related index patient, and 32 (65%) lived elsewhere. Persons living in the same household as the related index patient had the same prevalence regardless of whether they were (4 [33%] of 12) or were not (5 [36%] of 14) genetically related.

*T. whipplei* DNA in feces from family members ranged from 85 to 950,000 copies/g (mean ± SD 126,865 ± 296,176 copies/g); these numbers were significantly lower (p<0.001) than those of patients with active *T. whipplei* infections (range 170–6,400,000 copies/g [mean ± SD 2,410,000 ± 2,127,392 copies/g]). *T. whipplei* DNA in saliva ranged from 50 to 5,000 copies/mL (mean ± SD 2,400 ± 2,453 copies/mL) in family members and was lower than those of *T. whipplei*–infected patients (50–12,500 copies/mL [mean ± SD 3,639 ± 4,412 copies/mL]), but this difference was not significant (p = 0.5).

## Genotyping

Genotyping data were available for 5 families in which concentrations of *T. whipplei* DNA were high (Table 2). For 3 families, bacterial genotype was consistent between the patients and their families (genotypes 1, 3, and 19). Relatives from 2 of these families lived in the same household as the related index patient, whereas the relatives of the third family lived elsewhere. In 2 families, genotypes differed. In 1 family, the patient carried genotype 1, and his son had genotype 3; in the other, the patient carried genotype 82, his 2 nieces carried genotype 3, and his sister and mother carried a new genotype (83). For these 2 families, none of the relatives for whom a genotype was available lived in the same household as the index patient.

Overall, 52 different genotypes have been identified in France from 125 persons positive for *T. whipplei*, including family members. In the family of patient 1, genotype 1 was detected in all 3 members but in only 5 of 122 other persons; this difference was significant (p<0.001). In the family of patient 10, genotype 19 was identified in 2 of 2 members (p = 0.001) but in only 3 (2%) of the 123 other persons. In the family members of carrier 3, two of 5 persons carried a new genotype (83) that has not been previously reported (0/120) (p = 0.001). In the family of patient 11, genotype 3 was detected in 2 of the 2 members; outside of the family, it was observed in 31 (25%) of 123 other persons. This difference was not significant (p = 0.06). However, of the 31 persons with genotype 3, ten were children who previously had *T. whipplei*–associated gastroenteritis, and genotype 3 was suspected to be an epidemic clone among them (*14*).

## Western Blot Serologic Analysis

### Seroprevalence in the General Population of France

The overall seroprevalence for blood donors 18–66 years of age was 103 (52%) of 200 (Figure, panel A).

Table 1. Results of *Tropheryma whipplei* PCR on 74 relatives from 21 families of patients with classic Whipple disease, localized *T. whipplei* chronic infection, or asymptomatic carriers, France, 2003–2011

| *T. whipplei* | No. relatives (no. families) | No. female relatives | Age, all patients (mean ± SD) | No. samples PCR positive/no. tested (%) | | |
|---|---|---|---|---|---|---|
| | | | | Feces | Saliva | Feces or saliva |
| Overall | 74 (21) | 40 | 2 mo–79 y (38 y ± 22 y) | 24/64 (38) | 7/70 (10) | 25/74 (34) |
| Classic Whipple disease | 50 (13) | 29 | 2 mo–79 y (38.8 y ± 22 y) | 14/45 (31) | 4/48 (8) | 14/50 (28) |
| Localized infection | 14 (5) | 5 | 7–75 y (40.7 y ± 24.9 y) | 2/9 (22) | 1/12 (8) | 3/14 (21) |
| Carrier | 10 (3) | 6 | 8–65 y (29.6 y ± 21.1 y) | 8/10 (80) | 2/10 (20) | 8/10 (80) |

Table 2. *Tropheryma whipplei* genotyping for patients and their family members, France, 2003–2011*

| Study participant | PCR result | HVGS1 | HVGS2 | HVGS3 | HVGS4 | Genotype | Lived in household of index patient |
|---|---|---|---|---|---|---|---|
| Patient 1 family | | | | | | | |
| Patient 1 | + | 1 | 1 | 1 | 3 | 1 | Index patient |
| Father | + | 1 | 1 | 1 | 3 | 1 | No |
| Mother | + | 1 | 1 | 1 | 3 | 1 | No |
| Patient 10 family | | | | | | | |
| Patient 10 | + | 8 | 1 | 2 | 3 | 19 | Index patient |
| Husband | + | 8 | 1 | 2 | 3 | 19 | Yes |
| Son | – | NA | NA | NA | NA | NA | Yes |
| Daughter | – | NA | NA | NA | NA | NA | Yes |
| Patient 11 family | | | | | | | |
| Patient 11 | + | 1 | 6 | 1 | 1 | 3 | Index patient |
| Husband | + | 1 | 6 | 1 | 1 | 3 | Yes |
| Patient 7 family | | | | | | | |
| Patient 7 | + | 1 | 1 | 1 | 3 | 1 | Index patient |
| Son | + | 1 | 6 | 1 | 1 | 3 | No |
| Daughter | + | NA | NA | NA | NA | NA | No |
| Wife | – | NA | NA | NA | NA | NA | Yes |
| Carrier 3 family | | | | | | | |
| Carrier 3 | + | 1 | 1 | 25 | 3 | 82 | Index patient |
| Sister | + | 1 | 6 | 5 | 1 | 83 | No |
| Mother | + | 1 | 6 | 5 | 1 | 83 | No |
| Niece 1 | + | 1 | 6 | 1 | 1 | 3 | No |
| Niece 2 | + | 1 | 6 | 1 | 1 | 3 | No |
| Father | – | NA | NA | NA | NA | NA | No |
| Nephew 3 | – | NA | NA | NA | NA | NA | No |

*HVGS, highly variable genomic sequences; +, positive; –, negative; NA, not available.

Seroprevalence for patients hospitalized in the University Hospitals was comparable (Figure, panel B). However, the seroprevalence for children <4 years of age (5 [25%] of 20) was lower than in the overall population ≥4 years of age (35 [44%] of 80). Although this difference was not significant (p = 0.1), the seroprevalence for children <4 years of age was significantly lower than that of blood donors (103 [52%] of 200; p = 0.02).

### Serologic Analysis for Patients and their Families

Among patients and their families tested by using Western blot analysis, 8 patients with classic Whipple disease had deficient immune response to *T. whipplei*, whereas the 2 chronic carriers showed a strong immune response (online Appendix Table, wwwnc.cdc.gov/EID/article/18/06/11-1038-TA1.htm). Of the 3 patients who had localized *T. whipplei* infection, 2 had a deficient immune response, and the immune profile for 1 suggested carrier status. A positive *T. whipplei* response by Western blot occurred significantly more often in family members of patients or carriers (23 [77%] of 30) than in blood donors (103 [52%] of 200; p = 0.01) or control hospitalized patients (40 of 100; p<0.001). Of the 18 family members who were *T. whipplei* positive, 15 had a strong immune response. Of the 12 family members who were *T. whipplei* negative, 8 had a strong immune response. The 7 family members who had a deficient immune response to *T. whipplei* were from 3 different families.

## Discussion

Our data demonstrate that *T. whipplei* DNA is more prevalent in the feces and saliva of family members of patients with *T. whipplei* infection or asymptomatic carriage than in persons related by genetics or marriage. Family members were positive even if they were tested several months after their family member began antimicrobial treatment for *T. whipplei* infection. In addition, 8 of 10 persons who had close contact with chronic carriers were *T. whipplei* carriers. The comparable prevalence of this carriage among genetically related and non–genetically related relatives suggests that no genetic susceptibility exists to *T. whipplei*. The increasing prevalence of *T. whipplei* in the families seems linked to a bacterial exposure from the same source or reservoir.

The significant detection of the same bacterial genotypes in most families strongly supports the same origin of *T. whipplei* within a family. The different genotypes of *T. whipplei* within 2 families are not evidence against a common source or reservoir because *T. whipplei* has a wide heterogeneity (*4*,*22*). In 1 of the discrepant families, identification of a new genotype (83) among 2 relatives of the same family cannot clearly be linked to chance. Furthermore, the fact that relatives of 2 families with the same *T. whipplei* strain lived in the same household as the related index patient, whereas all the relatives with discrepant genotypes lived elsewhere, strongly suggest that those living together have a genetically more homogene *T. whipplei* strain than do those living elsewhere. Thus, the genotyping results and the high prevalence of *T. whipplei*

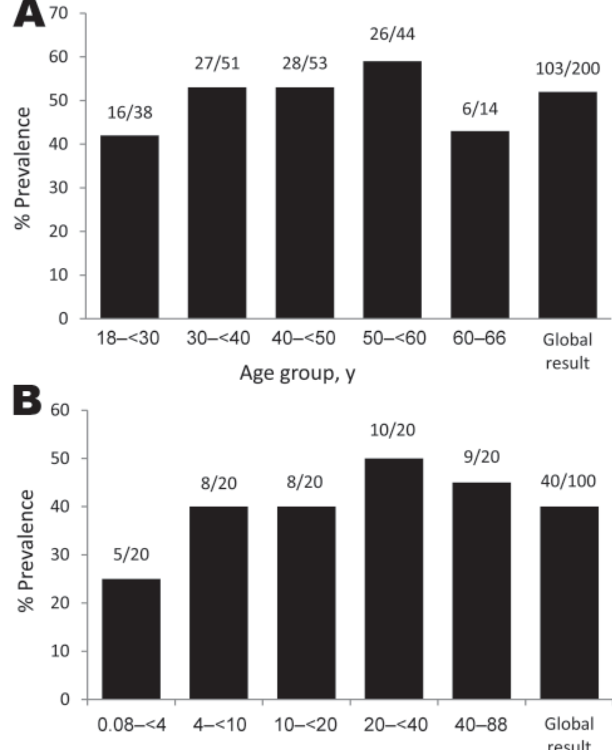


Figure. Seroprevalence of *Tropheryma whipplei* on the basis of Western blot serologic analysis of A) 200 serum samples from blood donor controls and B) 100 serum samples from patients hospitalized in University Hospitals, by age group, Marseille, France, 2003–2011.

in saliva and feces from family members of patients with *T. whipplei* infection or asymptomatic carriage indicate that relatives were more exposed than the general population to *T. whipplei*.

This exposition may be linked to a common source or reservoir. However, most (65%) relatives did not live in the same household and, in some instances, in the same city or region; they reported contact only during family gatherings. Also, the families did not always meet in the same place, which suggests that if the source of *T. whipplei* is common, it probably is linked to a human origin. Thus, our data strongly support the hypothesis that *T. whipplei* is transmissible between humans and is therefore contagious (Table 3). This possibility, first raised by a carrier, was initially considered unlikely. Later, the identification of clones circulating in France among children with gastroenteritis (*14*) and in western Africa (*27*) suggests this interhuman transmission. *T. whipplei* is known to be viable in feces and saliva from patients (*4*,*28*), suggesting that the bacterium might be transmitted through the fecal–oral (*2*) and oral–oral routes (*28*).

Overall, the seroprevalence in family members of patients was 77%, which is higher than in that of the general population of France (≈50%). These data show that relatives of patients or chronic carriers have more frequent contact than does the general population with *T. whipplei*. In addition, seroprevalence increased with the age: seropositivity in children <4 years of age occurred less often than in older children and adult blood donors. These data suggest that persons have contact with and seroconvert against *T. whipplei* most often during childhood and that about half of the population of France has been infected with *T. whipplei*. These results are consistent with our finding that 15% of hospitalized young children tested who have gastroenteritis have high fecal loads of *T. whipplei* (*14*). However, the seroprevalence in France is lower than in rural Senegal (72.8%), where the bacterium is highly prevalent (*27*). Taken together, these data confirm that *T. whipplei* is extremely common in our environment.

Our data highlight the role of host factors in Whipple disease. For example, for patient 1, the same genotype caused asymptomatic infections in the parents who had a strong immune response to *T. whipplei* but caused Whipple disease in their child who had a deficient immune response. The lack of detectable antibodies in serum indicates a defect in the immune response. The role of immunosuppression has been documented in the worsening of Whipple disease (*2*,*29*). The overall data from the serology of the patients and their families confirm that immune responses differ between patients and asymptomatic carriers (*23*–*25*). Immune reactivity is low in patients who have *T. whipplei* infections, whereas their family members who are asymptomatic carriers develop a strong immune response to *T. whipplei*. Thus, paradoxically, the deficient immune response by use of *T. whipplei* Western blot is the current tool to differentiate patients with classic Whipple disease from *T. whipplei* carriers. Finally, the 7 family members who lacked immune responses to *T. whipplei* were from the same 3 families. Of these persons, 2 children were carriers.

Table 3. Arguments for and against the intrafamilial transmission of *Tropheryma whipplei*, France, 2003–2011

| Argument in favor | Argument against |
|---|---|
| Epidemiologic | |
| *T. whipplei* carriage is significantly more common in persons related by genetics and marriage (34%) than in the general population of France (2%–4%) | Presence of few different bacterial genotypes in some families |
| Significant detection of the same bacterial genotypes in most families | |
| Most persons did not live in the same household and had contact only during family gatherings in different places | |
| Microbiologic | |
| Positive serologic results in relatives (77%) significantly higher than in the general population of France (48%) | |

The high prevalence of *T. whipplei* carriage in relatives raises several questions. Follow-up of these families will help to assess the risk for reinfection in patients successfully treated and without lifelong antimicrobial prophylaxis (*30*). In these households, 2 patients with localized chronic *T. whipplei* infection were reinfected after successful therapy. The need for systematic screening of relatives to propose a specific management will be also evaluated. We can suggest for relatives who report arthralgias the detection of *T. whipplei* by testing saliva and fecal specimens. Multiple factors may be necessary to observe the evolution from acute *T. whipplei* primary infection to chronic infection. Whipple disease is probably linked to a specific immune response to *T. whipplei* because the same genotype is responsible for various clinical manifestations and Whipple disease patients do show development of other infectious diseases. Another strong argument in favor of a specific defect in the immune response is the nature of lifetime susceptibility with relapse in patients with Whipple disease (*30*). We hypothesize that, similar to herpes virus encephalitis, a specific genetic defect might be involved in the development of Whipple disease (*2*,*31*,*32*).

Understanding of the natural history of *T. whipplei* continues to gradually increase. After contamination, including interhuman transmission, patients develop acute infection and may develop specific antibodies. Depending on host factors, patients eliminate *T. whipplei* and may harbor specific antibodies; carry it chronically for at least 5 years (D. Raoult, unpub. data) while exhibiting strong immune responses; or suffer from subacute or chronic infections, including classic Whipple disease without mainly developing antibody response.

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Dr Fenollar is a physician and research scientist working at the Unité des Rickettsies, Université de la Méditerranée, in Marseille. Her main research interests include *T. whipplei* and Whipple disease.

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# Human Gyrovirus DNA in Human Blood, Italy

Fabrizio Maggi, Lisa Macera, Daniele Focosi, Maria Linda Vatteroni, Ugo Boggi, Guido Antonelli, Marc Eloit, and Mauro Pistello

Human gyrovirus (HGyV) is a recent addition to the list of agents found in humans. Prevalence, biologic properties, and clinical associations of this novel virus are still incompletely understood. We used qualitative PCRs to detect HGyV in blood samples of 301 persons from Italy. HGyV genome was detected in 3 of 100 solid organ transplant recipients and in 1 HIV-infected person. The virus was not detected in plasma samples from healthy persons. Furthermore, during observation, persons for whom longitudinal plasma samples were obtained had transient and scattered presence of circulating HGyV. Sequencing of a 138-bp fragment showed nucleotide identity among all the HGyV isolates. These results show that HGyV can be present in the blood of infected persons. Additional studies are needed to investigate possible clinical implications.

In 2011, Sauvage et al. reported the discovery of a novel virus in human skin specimens and named it human gyrovirus (HGyV) (*1*). The characteristics of its genome—a single, closed molecule of circular, negative-sense DNA ≈2,300 nt long—and sequence homology with the chicken anemia virus (CAV) have suggested that HGyV might be the first human-infecting member of the genus *Gyrovirus*, which is part of the family *Circoviridae* and encompasses only 1 previously known species, CAV (*2*).

The genome of HGyV, which resembles CAV more closely than other members of the family (*1*,*3*), contains an untranslated region of ≈380 nt and 3 major partially overlapping open reading frames (named viral protein [VP] 1, VP2, and VP3) that encode proteins of 465, 231, and 31 aa, respectively. Whether HGyV and equivalent CAV proteins have similar functions is unknown. VP3 products of HGyV and CAV (for which the coded protein has been named apoptin) share short, functionally pivotal amino acid motifs, suggesting that HGyV also encodes an apoptin-like protein. The CAV apoptin induces tumor-specific apoptosis in a p53-independent fashion and has been shown to be a potential anticancer therapeutic agent in various animal models (*4*–*8*).

The epidemiology, biologic properties, and pathogenic potential of HGyV remain poorly understood. Sauvage et al. (*1*) detected the HGyV genome in nonlesional skin specimens of healthy persons and 1 HIV-positive patient but not in respiratory and fecal samples. This observation suggests that HGyV is most likely part of the normal skin microflora of humans, similarly to other recently discovered viruses (*9*,*10*). However, like related animal viruses, CAV infects a large range of cell types and causes a variety of pathologies (including bone marrow aplasia leading to aplastic anemia, hemorrhage, and lymphoid depletion) and increased death in young chicken (*11*). Also, CAV infection has been associated with the worsening of pathologies caused by other viral and bacterial agents (*11*–*13*).

Thus, because HGyV might cause clinically relevant disorders, guidance in choosing the directions for clinical investigation is crucial and needs to come from studies aimed at defining the prevalence of HGyV infection in different human populations, portal of entry, type of cells targeted during primary amplification, and site of latency/persistence. We investigated the presence of HGyV DNA in blood samples of 301 persons in Italy using specific PCRs. The results indicated overall HGyV positivity of 1.3%.

## Materials and Methods

### Patients and Samples

During December 2011, we studied 301 randomly selected persons living in central Italy. Most (251) were diseased patients whose blood samples had been submitted to the Virology Unit, Pisa University Hospital (Pisa,

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Italy), by local hospitals for routine virologic analysis; the remaining 50 were healthy blood donors. The patients comprised 151 HIV-infected persons (mean ± SD age 47 ± 14 years [range 18–80 years]; 115 men) who, before initiation of the study, had received no antiretroviral treatment. The patients also were examined for xenotropic murine-leukemia virus-related virus in a previous study (*14*). The other 100 patients (mean ± SD age 56 ± 8 years [range 36–69 years]; 71 men) were solid organ transplant recipients: 50 had received a liver transplant, and 50 had received a kidney transplant. Plasma samples were collected from patients on the day of transplant and then at selected times after transplant. Aliquots were prepared immediately, stored, and kept under sterile conditions at –80°C until use. Written informed consent was collected from each patient.

### HGyV DNA Detection

Viral DNA was extracted from 400 µL of peripheral whole blood or 200 µL of plasma by using the Maxwell 16 System (Promega, Madison, WI, USA) or QIAamp DNA blood kit (QIAGEN, Hilden, Germany), respectively, according to the manufacturers' instructions. Extracted DNA was amplified with 2 PCR protocols (developed and provided by V. Sauvage et al.), which target the VP1 gene of the viral genome.

The first amplification was a single-step TaqMan real-time PCR (rtPCR) designed on a 72-nt fragment. The assay was performed in a 25-µL volume containing 400 nmol/L of each primer (HGyV-rtFP: 5′-TGCTTGCAACAATGCCTTTAGA-3′; HGyV-rtRP: 5′-CCCTGCAAGTGCTGAGGATAA-3′), 200 nmol/L double-labeled probe (HGyV-rtP: 5′-FAM-CAAAGAGC AAAATCGGAGGCCCTAACC-TAMRA-3′) 5 µL extracted viral DNA, and the Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) containing deoxynucleoside triphosphate, and Taq DNA polymerase. Reaction was run in triplicate for each sample in an iQCYCLER rtPCR detection instrument (Bio-Rad Laboratories, Hercules, CA, USA) by using a standardized program (95°C 10 min; 45 cycles of 15 s at 95°C, and 60°C 60 s; and 40°C 30 s). A sample was considered rtPCR positive when HGyV DNA was detected in 2 of 3 replicas and when amplifications were specific as determined by 2% agarose gel electrophoresis.

A 178-bp fragment was amplified by a nested PCR format, described by Sauvage et al. (*1*) with modifications. This PCR was performed for 25 cycles with sense primer HGyV-OF (5′-CAAAATCGGAGGCCCTAACCC-3′) and antisense primer HGyV-OR (5′-ATGCCTGAATAGCTGC CAGCC-3′) under the following conditions: denaturation at 94°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 45 s. The product of this reaction (10 µL) was then re-amplified for 35 cycles with internal primers HGyV-IF (5′-GGTCAGCACAAACGACGCAG-3′) and HGyV-IR (5′- AGGTCTCCCATAGCGTCCAG-3′) at the same PCR conditions. The reactions were conducted in a 50-µL PCR mixture containing Taq DNA polymerase, each deoxynucleoside triphosphate at a concentration of 10 mmol/L, primers (20 µmol/L each), and optimized buffer components.

All samples were tested at least in duplicate and on different occasions. The amplified product was analyzed by electrophoresis on a 2% agarose gel after ethidium bromide staining. Amplicon size was compared with standard molecular mass markers. To minimize contamination risk, serum handling, DNA extraction, PCR amplification, and electrophoresis analysis were conducted in separated rooms. Negative controls were added during DNA extraction and PCR amplification. To validate the amplification process, positive controls (obtained from M.E.) were run in each PCR.

### Sequence Analysis

All HGyV PCR–positive isolates were characterized by sequencing a 138-bp fragment (from nt 1328 to 1465 of VP1 gene of the representative isolate FR823283) encompassing the target region of nested PCR. PCR amplicons, purified from the gel by using a QIAquick Gel Extraction Kit (QIAGEN, Chatsworth, CA, USA), were sequenced by using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and an automatic DNA sequencer (ABI model 3130; Applied Biosystems). Nucleotide sequences were aligned with the only sequence available at GenBank at the time of writing and by using the ClustalW algorithm included in BioEdit version 7.0.9.0 (www.clustal.org).

## Results

### Evidence of HGyV Infections

No samples from 50 healthy blood donors studied yielded positive results for HGyV (Table 1). Of 4 samples in which HGyV DNA was detected, 3 (6%) were from kidney transplant recipients and 1 (0.7%) was from an HIV-infected patient.

Table 1. Prevalence of human gyrovirus DNA in 251 HIV-positive or transplant recipient patients and 50 blood donors, Italy

| Group | Material tested | No. persons | No. (%) HGyV positive |
|---|---|---|---|
| Healthy persons | Plasma | 50 | 0 |
| HIV-positive patients | Whole blood | 151 | 1 (0.7) |
| Organ transplant recipients | | | |
| Liver | Plasma | 50 | 0 |
| Kidney | Plasma | 50 | 3 (6.0) |
| Total | | 301 | 4 (1.3) |

### Longitudinal Study of HGyV Viremia in Transplant Recipients

We examined plasma samples from 100 transplant patients for whom we had sequential samples obtained at selected times after transplant. Three of these patients who had received a kidney transplant tested positive for HGyV DNA, indicating that they had systemic HGyV infection. When additional samples of these patients were examined, a similar pattern emerged (Table 2). Plasma samples from 2 transplant recipients were already HGyV positive when they were first examined before transplantation. Subsequently, HGyV DNA detection was intermittent in the posttransplant samples: it was positive at month 12 (patient AL) and 6 (patient CV) but negative in the other samples tested. For patient MG, we examined 4 blood samples obtained before and after HGyV detection in plasma. At all these times, the plasma tested repetitively negative for HGyV DNA.

### Genetic Analysis of HGyV Isolates

Sequencing was conducted on all 6 PCR fragments obtained. All the isolates were related to the previously published strain, and the sequences obtained were virtually identical in the nucleotide fragment examined (online Appendix Figure, wwwnc.cdc.gov/EID/article/18/6/12-0179-FA1.htm). When blood specimens from the same patient were sequenced, no nucleotide change was noted among the viral sequence fragments obtained at any time.

## Discussion

The recent discovery of a human virus similar to CAV prompted an investigation of samples collected from patients and from healthy blood donors in central Italy. This investigation confirms that HGyV is present in humans, extends the previous findings, and raises several points. In the only study published, the HGyV genome had been investigated in 115 nonlesional skin specimens from adults and 138 specimens (46 nasopharyngeal aspirates and 92 fecal samples) from children. HGyV DNA was found in only 5 nonlesional skin specimens (*1*), suggesting that the virus could be a member of the human skin virome. In our study, plasma samples were taken from 251 immunocompromised patients (151 patients with HIV infection and 100 transplant recipients) and 50 healthy donors. HGyV DNA was demonstrated in the plasma of 4 persons, all with dysfunctional immune systems.

Table 2. Time course of human gyrovirus DNA detection in 3 kidney transplant patients, Italy*

| Months after transplant | Plasma HGyV DNA | | |
|---|---|---|---|
| | Patient AL | Patient CV | Patient MG |
| 0 | Positive | Positive | Negative |
| 1 | ND | Negative | Negative |
| 3 | ND | ND | Negative |
| 6 | Negative | Positive | Positive |
| 9 | ND | Negative | Negative |
| 12 | Positive† | Negative | ND |
| 15 | Negative | ND | ND |

*HGyV, human gyrovirus; ND, not determined.
†The urine sample tested at month 12 was HGyV negative.

The presence of HGyV in blood of infected humans suggests that the infection might also be systemic. The finding is not totally unexpected because CAV and the recently discovered avian gyrovirus 2, a virus genetically similar to HGyV, can circulate in the blood of infected animals (*15–18*). The clinical significance of HGyV viremia and relationship to induction of pathogenic processes is unclear. However, among the patients in whom virus was demonstrated, most had received a kidney transplant and thus had severe underlying nephropathy. The 3 kidney transplant recipients were a 52-year-old man with focal segmental glomerulosclerosis, a 21-year-old woman with lacrimoauriculodentodigital syndrome (i.e., Levy-Hollister syndrome), and a 57-year-old man with end-stage renal failure of unknown cause. All 3 recipients received basiliximab induction and triple maintenance immunosuppressive therapy with prednisone, mycophenolate mofetil, and cyclosporine A. The remaining HGyV-positive patient was a 32-year-old HIV-positive man who, when tested for HGyV, had an HIV load of 156,000 copies/mL and a CD4+ count of 465 cells/µL.

Data collected over time showed the occasional detection of viral DNA in blood. In fact, a similar pattern was observed in all the positive patients studied: plasma HGyV-positive samples alternated with virus-negative samples, indicating that circulating virus was intermittent. This finding also was confirmed in the HIV-positive patient, for whom the only additional plasma sample obtained 18 months after HGyV detection was HGyV negative (data not shown). Analysis of more data from additional studies is needed to understand the role of this transient detection of the virus, which might represent a putative short-lived acute infection with possible subsequent re-infection or just declines of the HGyV load under the lower limit of sensitivity of the detection methods used.

The limited size of the PCR fragment sequenced does not enable us to determine with certainty whether the HGyV DNA detected before transplantation and at later times were the same. This information could explain possible reinfections and/or persistence of the virus. Further molecular studies with larger fragments from variable regions of HGyV genome will be necessary to evaluate whether the virus persists in the infected host.

Dr Maggi is a clinical virologist at the Clinical Virology Unit of the Pisa University Hospital. His main interest is in natural history and immunopathogenesis of infection by viruses, particularly emerging viruses.

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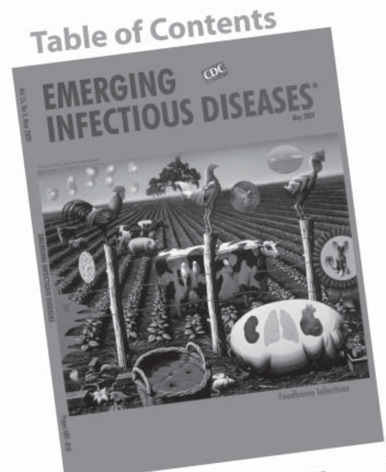


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# *Clostridium difficile* Infection, Colorado and the Northwestern United States, 2007[1]

Jennifer L. Kuntz, Eric S. Johnson, Marsha A. Raebel, Amanda F. Petrik, Xiuhai Yang, Micah L. Thorp, Steven J. Spindel, Nancy Neil, and David H. Smith

To determine the incidence of *Clostridium difficile* infection during 2007, we examined infection in adult inpatient and outpatient members of a managed-care organization. Incidence was 14.9 *C. difficile* infections per 10,000 patient-years. Extrapolating this rate to US adults, we estimate that 284,875 *C. difficile* infections occurred during 2007.

*Clostridium difficile* infection is a major source of illness in the United States (*1*). Population-based estimates of its incidence tend to include only subsets of infections defined by the setting of *C. difficile* acquisition (*2–4*) or are from patient populations outside the United States (*5–9*). These studies (*2–9*) offer useful data for control measures but do not help clinicians and policy makers understand the population-based incidence of *C. difficile* infection. To determine the incidence of *C. difficile* infection during 2007, we estimated the incidence of *C. difficile* infection among members of 2 Kaiser Permanente health plans and extrapolated our incidence estimate to the US adult population.

## The Study

We identified *C. difficile* infections during January 1–December 31, 2007, among Kaiser Permanente Colorado and Kaiser Permanente Northwest members ≥20 years of age. The health plans had a combined membership of ≈900,000 on any given day during 2007. We collected patient membership, demographic, and clinical data using electronic databases. *C. difficile* infections were identified through International Classification of Diseases, 9th Revision (ICD-9), code 008.45 ("Intestinal infection due to *C. difficile*") recorded during an inpatient or outpatient health care visit or a positive *C. difficile* toxin test result. To increase the likelihood that cases were symptomatic, we further required that positive toxin test results be associated with dispensation of metronidazole or vancomycin in the outpatient pharmacy in the 7 days before or after a positive test result. Specimens were reported as negative or positive on the basis of results from a Meridian Premier Toxin A/B enzyme immunoassay (Meridian Bioscience, Cincinnati, OH, USA).

A *C. difficile* infection was considered incident if the patient did not have a history of a *C. difficile* diagnosis, a positive toxin test result, or an outpatient prescription for vancomycin or metronidazole in the previous 180 days. To ensure cases were incident and to collect baseline characteristics, patients with *C. difficile* infections were required to have continuous membership and prescription drug coverage for 1 year before the date of *C. difficile* infection.

We calculated the total incidence of *C. difficile* infection as the number of incident cases among persons ≥20 years of age per 10,000 person-years of observation. Age- and sex-specific incidence rates were also calculated. A patient could have had >1 incident *C. difficile* infections if they occurred >180 days apart. Denominator data were based on duration of membership for persons ≥20 years of age with continuous membership and prescription drug coverage for 1 year before July 31, 2007. To project the national incidence of *C. difficile* infection, we applied pooled, age-specific incidence, and sex-specific incidence estimates to the 2007 US population. As a sensitivity analysis, we also provided the incidence projection for US whites because earlier surveys of members showed a predominantly (90%) white membership, and race data were unavailable for a substantial proportion of members.

We identified 870 incident *C. difficile* infections among members ≥20 years of age in 2007; a total of 473 (54%) of 870 *C. difficile* infections were identified among outpatients. Overall incidence was 14.9 *C. difficile* infections per 10,000 patient-years; age-specific incidence rates ranged from 2.4 infections per 10,000 patient-years for men 20–29 years of age to 87.1 infections per 10,000 patient-years for men ≥80 years of age (Table). On the basis of these age- and sex-specific rates, we estimated that 284,875 *C. difficile* infections occurred among the overall

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US population ≥20 years of age. We also estimated that 241,815 infections occurred among US whites (Table).

## Conclusions

Our study identified new infections among this managed care population in 2007 and estimated the occurrence of new infections within the US population. Our approach differed from those of previous reports that were based on individual medical institutions, hospital discharge databases, or voluntary surveillance in targeted populations and geographic areas (*10–13*). We differentiated between incident and prevalent infections. Identification of incident infections is needed to understand the causes and predictors of infection and to develop clinical interventions to prevent infection. Because we also identified *C. difficile* infection among inpatients and outpatients, our estimates more fully account for total *C. difficile* infection and provide a foundation for studying the potential spread of *C. difficile* between ambulatory and hospitalized persons.

Although different objectives and methods make direct comparisons difficult, we found that the incidence of *C. difficile* infection among the Kaiser Permanente population studied (14.9 infections/10,000 patient-years) did not differ substantially from the 11.2 *C. difficile*–associated hospitalizations among adults per 10,000 population identified in the 2005 Nationwide Inpatient Sample (*14*). Our estimate of 240,000–285,000 incident *C. difficile* infections in the US adult population in 2007 is comparable with the results of an extrapolation by Campbell et al., which found that on the basis of health care–facility surveillance data, 333,000 initial and 145,000 recurrent *C. difficile* infections might have occurred nationwide in 2006 (*15*). Although previous reports of inpatient encounters resulting from *C. difficile* infection or total *C. difficile* infections are needed to describe the impact of *C. difficile* infection on the health care system, our identification of infections in inpatient and outpatient health care settings may provide a more accurate estimate of *C. difficile* infection incidence.

Many discharge databases do not include longitudinal patient identifiers; even though these databases can identify diagnoses during hospitalizations, they cannot link hospitalizations to specific patients. Thus, multiple hospitalizations for recurrent or refractory *C. difficile* infection would count as multiple infections, whereas we followed-up patients over time to ensure that only incident infections were counted. Furthermore, although we relied on ICD-9 codes to identify cases among inpatients, we also used *C. difficile* toxin tests, treatment for *C. difficile* infection, and *C. difficile*–related health care encounters to identify infections in outpatients. We could have missed infections in inpatients if the ICD-9 code for *C. difficile* was absent; however, our use of toxin test results and pharmacy dispensing records likely resulted in more accurate and complete identification of *C. difficile* infection among outpatients. In fact, we found that >95% of patients with positive toxin test result had a treatment-dispensing or ICD-9 code for *C. difficile* infection.

Our data might overestimate or underestimate the incidence of *C. difficile* infection or affect the interpretation of our results in 4 ways. First, our population was predominantly white. Although we are unaware of any evidence that *C. difficile* infections occur disproportionately by race or ethnicity, we projected our incidence rate to the entire and white-only US populations to acknowledge the distribution of race in the study population. Second, our insured population could be healthier than the US population. Third, Kaiser Permanante has policies and procedures that promote judicious prescription of antimicrobial drugs and effective infection control and prevention. Collectively, a healthy population, health plan

Table. Incidence of *Clostridium difficile* infection among members ≥20 years of age of Kaiser Permanente Colorado and Northwest, USA, with projections to US white and US total populations, 2007*

| | Kaiser Permanente populations | | Projected no. US infections | |
|---|---|---|---|---|
| Sex/age, y | Incident infections, no. | Incidence rate* (SE) | Whites | Total |
| Female | | | | |
| 20–29 | 12 | 3.1 (0.91) | 4,920 | 6,383 |
| 30–39 | 31 | 6.1 (1.09) | 9,399 | 12,186 |
| 40–49 | 45 | 7.2 (1.1) | 13,079 | 16,386 |
| 50–59 | 80 | 11.5 (1.3) | 18,892 | 23,046 |
| 60–69 | 96 | 21.3 (2.2) | 23,340 | 27,681 |
| 70–79 | 119 | 42.0 (3.8) | 32,141 | 37,503 |
| ≥80 | 140 | 79.5 (6.7) | 42,559 | 48,015 |
| Male | | | | |
| 20–29 | 8 | 2.4 (0.85) | 3,969 | 5,009 |
| 30–39 | 18 | 3.9 (0.93) | 6,318 | 7,871 |
| 40–49 | 29 | 5.2 (0.96) | 9,274 | 11,321 |
| 50–59 | 62 | 10.1 (1.3) | 16,242 | 19,359 |
| 60–69 | 67 | 16.7 (2.0) | 16,747 | 19,355 |
| 70–79 | 73 | 31.1 (3.6) | 18,994 | 21,780 |
| ≥80 | 90 | 87.1 (9.2) | 25,941 | 28,980 |
| All | 870 | 14.9 (0.5) | 241,815 | 284,875 |

*Per 10,000 patient-years

policies, and clinician awareness might result in fewer *C. difficile* infections among this population than is observed in other health care settings. Fourth, toxin tests are imperfect, potentially leading to overestimation of incidence.

Our study provides population-level estimates of *C. difficile* infection in inpatients and outpatients. However, more efficient and timely methods for identifying and reporting *C. difficile* infection are needed to further improve understanding of the epidemiology of *C. difficile* infection and the interventions necessary to prevent them.

**Acknowledgment**

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This study was funded by Sanofi Pasteur.

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# Severe Fever with Thrombocytopenia Syndrome Virus, Shandong Province, China

**Li Zhao, Shenyong Zhai, Hongling Wen, Feng Cui, Yuanyuan Chi, Ling Wang, Fuzhong Xue, Qian Wang, Zhiyu Wang, Shoufeng Zhang, Yanyan Song, Jun Du, and Xue-jie Yu**

Severe fever with thrombocytopenia syndrome, which results in severe illness and has a high case-fatality rate, is caused by a novel bunyavirus, severe fever with thrombocytopenia syndrome virus. We found that samples from 2/237 (0.8%) healthy persons and 111/134 (83%) goats in Yiyuan County, Shandong Province, China, were seropositive for this virus.

Severe fever with thrombocytopenia syndrome (SFTS) is a serious infectious disease with 12% case-fatality rate that has been documented in 6 provinces rural in northeast and central China. SFTS is caused by a novel bunyavirus, SFTS virus (SFTSV) (*1*). The major clinical signs and symptoms of SFTS are fever, thrombocytopenia, leukopenia, and elevated serum hepatic enzyme levels.

SFTSV is classified in the family *Bunyaviridae*, genus *Phlebovirus*, and is believed to be transmitted by ticks because the virus has been detected in *Haemaphysalis longicornis* ticks (*1*). However, the disease can also be transmitted from person to person through contact with infected patient's blood or mucous (*2*,*3*). SFTSV seroprevalence in the human population is unknown, and the natural reservoir hosts of SFTSV have not been determined. We report results from a SFTSV serosurvey conducted on healthy persons and goats in Yiyuan County in Shandong Province, China, an area to which SFTS is endemic.

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## The Study

Yiyuan County, located in eastern China (117°48′–118°31′E, 35°55′–36°23′N; Figure), has an area of 1,636 $km^2$ and a population of 550,000 persons; 85% of the population is considered involved in agriculture. The county consists of low-lying hills with forests and grasslands and considered in a warm temperate zone, with a continental monsoon humid climate and 4 distinct seasons. The annual average temperature is 11.9°C, and the average rainfall is 720.8 mm. Farmers plant crops and fruit trees and raise goats as a major source of income in the area.

Most farm families have a herd of goats, and some of them have dogs. We found that goats and dogs in this county were heavily infested with ticks, with several hundred ticks found on each goat and dog. A recent serosurvey of domestic animals in Jiangsu Province found an SFTSV antibody positive rate of 57% in goats, 32% in cattle, 6% in dogs, 5% in pigs, and 1% in chickens (*4*). We selected goats for our seroprevalence survey because there was

Figure. Location of the villages (gray shading) in Yiyuan County, Shandong Province, China, where human and goat serum samples were collected in study of severe fever with thrombocytopenia syndrome seroprevalence. Maps at bottom show location of Yiyuan County in Shandong Province (left) and Shandong Province in China (right).

large population (n = 400,000) in Yiyuan County in 2011 (*5*) and because goats were heavily infested with ticks. Dogs were not surveyed because the small population of dogs made it difficult to obtain adequate sample numbers. Other domestic animals in the area (e.g., cattle, pigs, rabbits, and chickens) were not surveyed because they were usually raised in captivity.

The investigation of SFTSV seroprevalence was conducted during June 2011 in 10 rural villages in northwestern Yiyuan County with a total registered population of 7,406 (Figure). We recruited a convenience sample of 237 healthy volunteers from these villages and collected blood samples from all volunteers. The volunteers consisted of a small portion (3.2%) of the farmer population in the villages. A standardized questionnaire was used to obtain information on age, sex, history of illness, tick exposure, and occupation of each participant. All study participants were goat farmers who were also involved in agriculture and were longtime village residents. The research protocol was approved by the human bioethics committee of Shandong University, and all participants provided written informed consent.

Participant age ranged from 20 to 80 years (median 54 years); 150 (63%) were female. No volunteer was <20 or >81 years of age. The age and sex distribution of the study population may have resulted from the migration of men and young people from rural areas to cities or lower participation among older and younger person.

Serum samples were tested for total antibodies (IgG and IgM) to SFTSV by using a double-antigen sandwich ELISA kit, provided by Jiangsu Province Center for Disease Control and Prevention (*5*). The ELISA kit used recombinant nucleoprotein (NP) of SFTSV as an antigen, which was coated onto a plate. In the preliminary experiment, undiluted serum (50 μL) was added to a well of the plate, and the plate was incubated for 30 min at 37°C to enable SFTSV antibodies to bind to NP of SFTSV antigen. After washing, the bound SFTSV antibodies were reacted with horseradish peroxidase–labeled recombinant SFTSV NP and detected by substrates for horseradish peroxidase.

Absorbance of the plate was read at 450 nm. A sample was considered positive to SFTSV when the absorbance of the serum sample was ≥2.1× the absorbance of the negative control (provided by the manufacturer), which was 3 SD above the mean optical density at 450 nm for the persons sampled. The ELISA had similar specificity and sensitivity to the microneutralization assay and exhibited no cross-reactivity with hantavirus or dengue virus antibodies (*4*)

ELISA detected SFTSV antibodies in 3 healthy persons when undiluted serum samples were used. The positive samples were diluted to determine the antibody titers, and 2 serum samples were positive after dilution (Table 1). This finding could indicate a false-positive result for the serum that became negative upon dilution. Therefore, only the 2 persons whose samples remained positive after dilution were considered seropositive for SFTSV. Thus, the seroprevalence of SFTSV in the investigated population was 0.8% (2/237) (Table 1). Both of these persons were female, and neither reported SFTS symptoms in the past, being hospitalized for any clinically similar disease, or contact with a person who had fever and thrombocytopenia syndrome as defined (*1*).

Table 1. Age distribution of anti–severe fever with thrombocytopenia syndrome virus IgG/IgM detected in healthy human volunteers from rural area of Yiyuan County, Shandong Province, China

| Age group, y | No. persons tested | No. seropositive by ELISA (titer) |
|---|---|---|
| 0–20 | 0 | 0 |
| 21–30 | 4 | 1 (128) |
| 31–40 | 19 | 0 |
| 41–50 | 77 | 0 |
| 51–60 | 76 | 1 (512) |
| 61–70 | 34 | 0 |
| 71–80 | 27 | 0 |
| ≥81 | 0 | 0 |
| Total | 237 | 2 |

We also collected blood samples from 134 goats from 16 herds in 8 villages in the county during June and August 2011. Most famers in the area have goats, but few volunteered to donate their goats' blood for our study. Therefore, we selected all 16 herds whose owners allowed us to sample their goats and randomly sampled 2–20 goats from each herd (the number of goats sampled was determined by the owner). The goat serum samples were tested for total antibodies to SFTSV by using the double-antigen sandwich ELISA kit as described human samples. Of the 134 goats sampled, 111 (83%) had antibodies to SFTSV according to the established cutoff of ≥2.1× the absorbance of the negative contro. We further diluted the goat serum in 2-fold increments, from 1:8 to 1: 512, and the titers were all ≥32 (Table 2).

## Conclusions

We found a high seroprevalence of SFTSV among goats in Yiyuan County but a low seroprevalence among humans. The seropositive persons were from 2 different villages and were not linked to each other; only 1 was involved in herding goats and neither in milking or butcher-

Table 2. Anti–severe fever with thrombocytopenia syndrome virus IgG/IgM detected in serum samples from healthy goats from rural area of Yiyuan County, Shandong Province, China

| ELISA titer | No. positive serum samples |
|---|---|
| 32 | 9 |
| 64 | 24 |
| 128 | 25 |
| 256 | 10 |
| ≥512 | 43 |
| Total | 111 |

ing. Thus, the exposure route was not clear. Neither of the seropositive persons reported clinical illness; however, this finding is limited by the possibility of recall bias.

A previous study of 200 serum samples collected from SFTSV-endemic areas showed no subclinical infection with SFTSV (*1*). The discrepancy between that study and our study may be a result of the low overall seroprevalence of SFTSV. We conclude that subclinical SFTSV infections or a relatively mild form of SFTS illness may occur in humans; however, more research is needed.

### Acknowledgements

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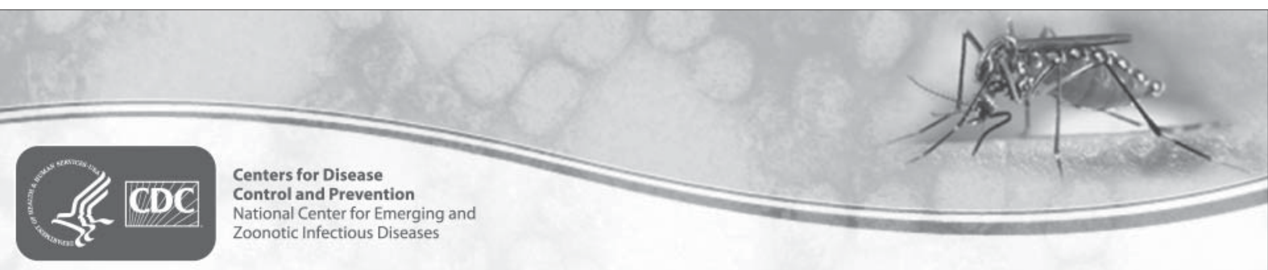


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# Macrolide-Resistant *Bordetella pertussis* Infection in Newborn Girl, France

**Sophie Guillot, Ghislaine Descours, Yves Gillet, Jérome Etienne, Daniel Floret, and Nicole Guiso**

A macrolide antimicrobial drug was administered to a newborn with cough. On day 23 of hospitalization, macrolide-resistant *Bordetella pertussis* was isolated from nasopharyngeal aspirates. DNA sequencing and PCR–restriction fragment length polymorphism showed a 2047 A-to-G mutation in the 3 copies of the 23S rRNA gene. Monitoring for macrolide resistance is essential in infants <6 months of age.

*Bordetella pertussis,* the causative agent of whooping cough, continues to circulate among children and adolescents even in regions with high vaccine coverage. Antimicrobial drug treatment contributes substantially to controlling transmission of the disease. In France, the treatment of choice is clarithromycin or azithromycin, which eliminate the bacterium from the respiratory tract of the infected patient and their close contacts (*1*). To date, erythromycin resistance in *B. pertussis* has been described only in the United States (*2*–*4*). The erythromycin-resistant *B. pertussis* isolates in the United States carry an A-to-G transition at nucleotide position 2047 of the 23S rRNA gene, in a region critical for erythromycin binding.

## The Study

We report the case of an 18-day-old girl, born without complication after 39 weeks of pregnancy, weighing 3,510 g. She was brought to the emergency department of Hopital Femme Mère Enfant (Lyon, France) with a 2-day history of cough without fever and worsening status. She had not previously been ill. The mother reported a cough that had persisted for 2 weeks. Physical examination found no fever, a heart rate of 169 beats/min, a respiratory rate of 60 breaths/min, signs of retraction, and an oxygen saturation of 92% in room air. Lung examination found rales in both basal fields, with a loose cough. The leukocyte count was 26.8 cells/mm$^3$, with 51% lymphocytes. Venous blood gas measurement showed a pH of 7.30, a carbon dioxide partial pressure of 7.19 kPa, and a partial oxygen pressure of 4.14 kPa. Chest radiograph showed thoracic distension without signs of condensation. Nasopharyngeal aspirates were positive for *B. pertussis* by culture and specific PCR (Cepheid, Maurens-Scopont, France).

The patient was admitted to the intermediate care unit and was given azithromycin for 3 days, but her condition worsened (respiratory rate of 90–100 breaths/min, transient episodes of bradycardia at 75–100 beats/min). She was referred to the pediatric intensive care unit (ICU) 4 days after admission. On day 5, the patient underwent a 300-mL volume exchange transfusion because of increasing oxygen requirement, despite continuous positive airway pressure (fraction of inspired oxygen 50%) and increased leukocyte count (64 cells/mm$^3$). The postexchange leukocyte count fell to 20 cells/mm$^3$, but the respiratory benefit was only moderate. On day 14, the leukocyte count had increased to 54 cells/mm$^3$, and the oxygen requirement to fraction of inspired oxygen 40%; a second 300-mL volume exchange transfusion was administered. A second sample of nasopharyngeal aspirates was obtained and was positive for *B. pertussis* by culture and specific PCR. Clarithromycin treatment was started on day 18 and continued for 7 days.

The patient started to improve after 23 days and was transferred from the ICU to the pulmonary unit on day 35. A routine nasopharyngeal aspirate was taken, and an erythromycin-resistant strain of *Staphylococcus aureus* was cultured from this aspirate ($10^5$ CFU/mL); cotrimoxazole was then administrated for 8 days. The patient recovered normal pulmonary function and was discharged on day 44. Cultures of the sample taken at discharge showed a single colony of *B. pertussis*, and clarithromycin was administered for 5 days. The patient recovered completely, and results of *B. pertussis*–specific culture and PCR with nasopharyngeal aspirates were negative for *B. pertussis* on day 98.

Three isolates were collected, one at admission (FR4929), one 18 days after azithromycin treatment (FR4930) and a third 19 days after clarithromycin treatment (FR4991). FR4929 and FR4930 were sensitive to all macrolides tested (erythromycin, clarithromycin, azithromycin), but FR4991 was resistant. The Etest MIC was >256 µg/mL for all macrolides. To our knowledge, macrolide resistance has not been found in other *B. pertussis* isolates collected in France since the late 1940s.

The 3 isolates and the erythromycin-resistant isolate A228 (*2*) from the United States were analyzed for biochemical characters and by pulsed-field gel electrophoresis, genotyping and expression of virulence as described in Bouchez et al. (*5*). The 3 isolates from France belong to the same group (group IV) as that of all

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*B. pertussis* isolates circulating in France since 1998 and A228. They all harbor a *PtxP3* allele, a *PtxA* allele, and a *prn* 2 allele, and expressed all tested virulence factors: pertussis toxin, adenylate cyclase–hemolysin, filamentous hemagglutinin, pertactin, and fimbrial protein type 3.

PCR amplification and DNA sequencing were used to determine whether the resistance to erythromycin was associated with a mutation in the 23S rRNA gene as described by Bartkus et al. (*2*). The sequences of ≈400 nt of the 521-bp PCR fragment were determined for the 3 isolates obtained from this patient, A228 (*2*), and the reference strain Tohama (*6*). An A-to-G mutation was found at position 2047 in the 23S rRNA gene in the genomes of FR4991 and A228 but not in the genomes of FR4929 and FR4930.

*B. pertussis* carries 3 copies of the 23S rRNA gene. To test whether all 3 copies were mutated, DNA amplified by PCR (which amplified the sequences of all 3 copies of the 23srRNA) from each isolate was digested with *BbsI*: the A2047G transition in the rRNA gene is predicted to create a *BbsI* restriction site (Figure). *BbsI* cleaved the entire DNA amplified from the erythromycin-resistant isolate FR4991, confirming the presence of a G at position 2047 in the 3 copies of the 23S rRNA gene. *BbsI* did not cleave the DNA amplified from the erythromycin-sensitive isolates FR4929 and FR4930 indicating that they have no A2047G mutation in any of the 3 copies of the 23S rRNA gene.

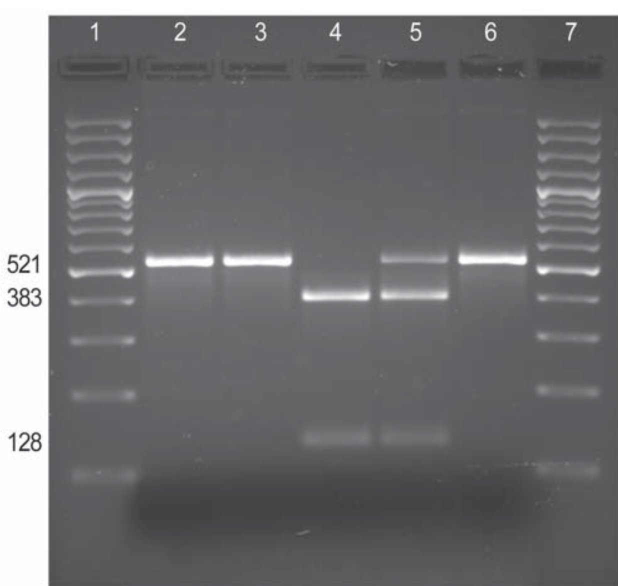


Figure. Screening for the A2047G mutation by PCR–restriction fragment length polymorphism analysis. The 521-bp fragment of the 23S rDNA gene amplified by PCR from the *Bordetalla pertussis* clinical isolates (FR4229, FR4930, and FR4991) and controls (A228 and Tohama I) was digested with the endonuclease BbsI. Lanes 1 and 7, M, 100-bp ladder (SM0321; Fermentas, St. Leon-Rot, Germany); lane 2, *B. pertussis* FR4929; lane 3, *B. pertussis* FR4930; lane 4, *B. pertussis* FR4991; lane 5, control *B. pertussis* A228 (erythromycin resistant, heterozygous); lane 6, control *B. pertussis* Tohama (erythromycin susceptible).

## Conclusions

The patient was brought for treatment with severe whooping cough which required ICU management and 2 courses of blood exchange. Nevertheless, *B. pertussis* resistance to macrolides is unlikely to be the cause of this severity because the infecting strain was found twice to be sensitive to all macrolides, and acquired resistance subsequently. However, despite appropriate treatments, we could not eradicate *B. pertussis*, and cultures were positive after 2 courses of macrolides and 1 course of cotrimoxazole given for an associated staphylococcal infection. Presumably, this prolonged carriage favored the acquisition of resistance.

This observation confirms the need for maintaining the ability to culture *B. pertussis* isolates to analyze the evolution of their characteristics as well as their antimicrobial drug resistance. Monitoring for macrolide resistance is thus essential when investigating individual treatment failures, in particular, in infants <6 months of age. The resistance mechanism we report is similar to that described for an isolate collected in the United States, but these findings do not rule out the potential for the emergence of alternative resistance mechanisms.

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Ms Guillot is assistant director of the French National Center of Reference of Whooping Cough and Other Bordetelloses and engineer in the Molecular Prevention and Therapy of Human Diseases Unit, Institut Pasteur, Paris. Her research interests focus on the molecular evolution of *Bordetella* in relation to clinical and public health problems, comparative genomic analysis, and development of tools to analyze gene expression.

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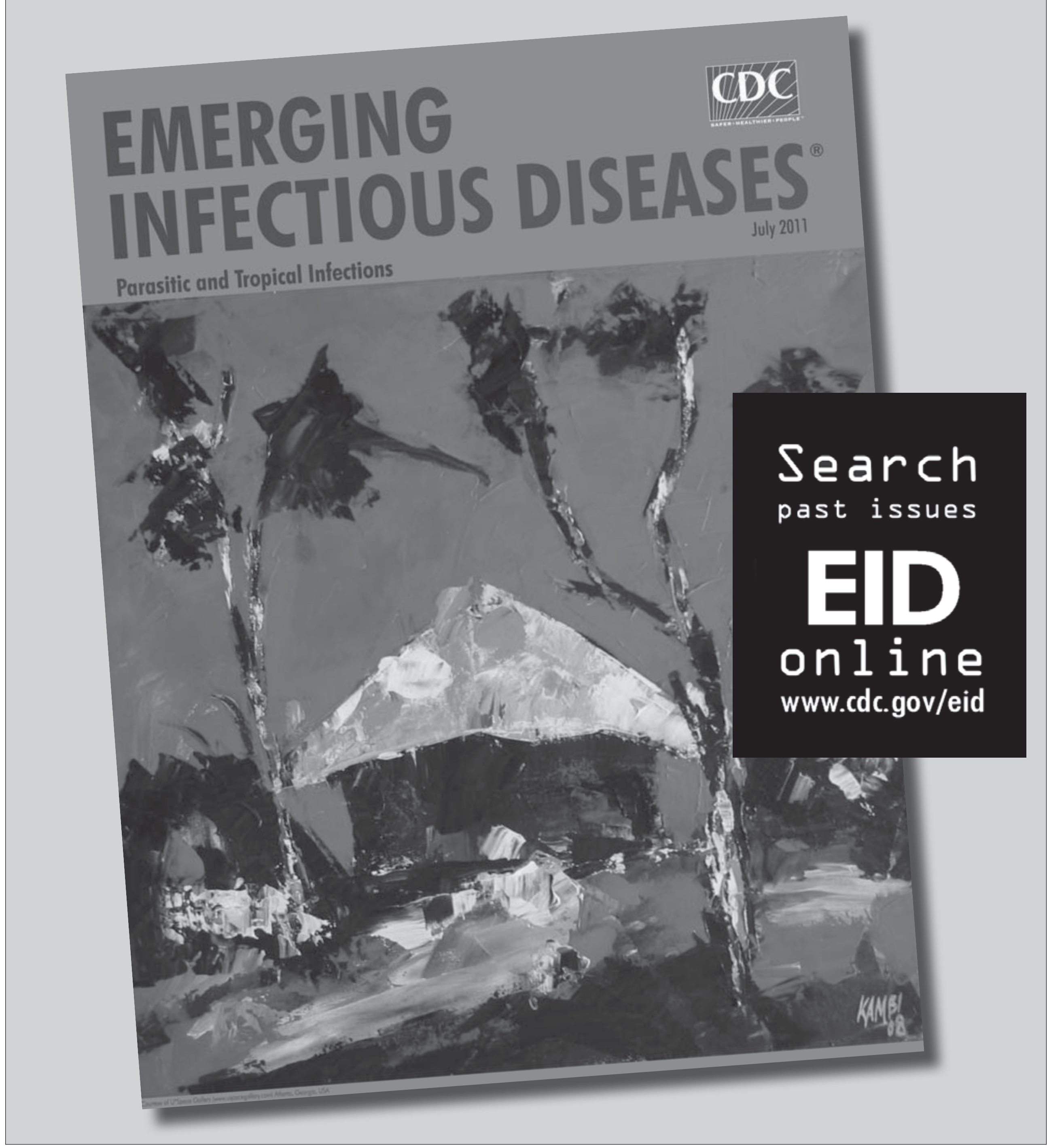

# Genome Analysis of Rift Valley Fever Virus, Mayotte

Catherine Cêtre-Sossah, Hervé Zeller, Marc Grandadam, Valérie Caro, François Pettinelli, Michèle Bouloy, Eric Cardinale, and Emmanuel Albina

As further confirmation of a first human case of Rift Valley fever in 2007 in Comoros, we isolated Rift Valley fever virus in suspected human cases. These viruses are genetically closely linked to the 2006–2007 isolates from Kenya.

Identified during the 1930s in Kenya, Rift Valley fever (RVF) is a zoonotic disease that circulates in many African countries and in the Arabian Peninsula (*1*,*2*). RVF virus (RVFV) epizootics are characterized by large sweeping abortion storms and substantial death rates in adult livestock (primarily sheep, goats, and cattle), with the death rate for newborn animals approaching 100% (*3*). Humans typically are infected by bites of infected mosquitoes or by percutaneous or aerosol exposure to contaminated fluids from infected animals. In most human cases, RVF is characterized by a self-limiting febrile illness (2–5 days), which progresses to more serious complications (hepatitis, encephalitis, blindness, or hemorrhagic syndrome) in only 1%–2% of infected persons (*4*,*5*). A large epizootic–epidemic occurred during 2006–2007 on the eastern African mainland, predominantly in Kenya (*6*) and Madagascar, during 2 successive rainy seasons (*7*).

In July 2007, a 12-year-old boy with a 2-month history of severe encephalitis was transferred from the Grande Comore, Union of the Comoros, to Mayotte (*8*,*9*). RVF infection was confirmed by IgM serologic analysis. Because of the proximity of Comoros and Mayotte, the RVF situation among humans in Mayotte was assessed. In serum samples from 7 humans with dengue-like syndromes, RVFV IgM or RVFV RNA was detected. We report the isolations and full sequence analysis of 2 RVF viral isolates from these serum specimens.

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## The Study

During January–April 2007, seven patients native to Mayotte were admitted to the hospital for severe dengue-like syndromes. Two patients were RVF seropositive by IgM and IgG, and the other 5 were positive by RVFV-specific reverse transcription PCR (RT-PCR) as detailed in Sissoko et al. (*9*). As described for other viruses, we used in-house IgM-capture enzyme immunoassays and in-house direct detection for IgG by using microplates coated with RVFV antigen and specific binding by using a peroxidase-labeled goat anti-human IgG conjugate (*10*).

RVFV isolates were obtained on Vero E6 cells from the serum of 2 hospitalized patients (serum collected on February 21 and March 20, 2008). RNA extracted by using the RNaid Kit (Qbiogene, Carlsbad, CA, USA) was reverse transcribed by PCR and amplified by using SuperScript One-Step RT-PCR with platinum Taq kit (Invitrogen, San Diego, CA, USA) with primers targeting the small, medium, and large segments (adapted from [*11*]). Overlapping RT-PCR fragments were purified by ultrafiltration. Sequencing reactions were performed by using the Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequence chromatograms from both strands were obtained on automated sequence analyzer ABI3730XL (Applied Biosystems). For sequence analysis, contig assemblies and sequence alignments were performed by using BioNumerics version 6.6 (Applied-Maths, Sint-Martens-Latem, Belgium).

We used 2 methods for phylogenetic reconstruction: maximum likelihood and the Bayesian inference. The best models of nucleotide substitution for each dataset were selected from the uncorrected and corrected Akaike Information Criterion, the Hannan and Quinn performance-based decision theory and Bayesian Information Criterion of Jmodeltest version 0.1 and TREEFINDER version October 2008 (Munich, Germany, distributed by its author at www.treefinder.de). The consensus substitution models proposed by the different software packages were selected for further analyses. Comparison of the maximum-likelihood method implemented by the TREEFINDER program with others was performed on the small, medium, and large segments by using the neighbor-joining and maximum parsimony methods from Mega5 software and the Bayesian approach by using MrBayes version 3.0B4 for phylogenetic reconstruction with random starting trees and run for 2,000,000 generations, sampling the Markov chains at intervals of 100 generations (*12*,*13*). Branch support values were obtained by using nonparametric bootstrapping with 1,000 resampling for PhyML and TREEFINDER and the posterior probabilities for the Bayesian approach estimated on 10,000 samples (sample frequency set to every 100th generation by using the Markov Chain Monte Carlo sampling). We compared topologies of the

maximum-likelihood and Bayesian trees obtained for the different segments.

The complete genome sequences performed on 2 human RVFV isolates from Mayotte referenced as 2008/00099 and 2008/00101 (deposited in GenBank/EMBL under accession nos. HE687302–HE687307) are embedded within the larger 2006–2007 East African clade, specifically within the lineage previously termed Kenya-1 (online Appendix Table, wwwnc.cdc.gov/EID/article/18/6/11-0994-TA1.htm). The Kenya-1 virus lineage includes 18 isolates, 8 human isolates (035/07Baringo Kenya 2007, SPU10315 Kenya 2007, Garissa 004/006 Kenya 2006, Dod002/007Tanzania 2007, 3162 Madagascar 2008, 3163 Madagascar 2008, 3164 Madagascar 2008, 3165 Madagascar 2008), 2 mosquito isolates (KLFMsq091/07 Kenya 2007, 131B04/06Garissa Kenya 2006), and 8 livestock isolates (1602Mombassa Kenya 2007, 2820Garissa Kenya 2007, 3644Baringo Kenya 2007, 473Kajaido Kenya 2007, 0611Kenya 2007, 3168 Madagascar 2008, 3169 Madagascar 2008, 3170 Madagascar 2008). The Kenya 2 virus lineage comprises 3 human isolates (1811Garissa Kenya 2006, 0094 Kenya 2007, Tan001/007 Tanzania 2007).

Because maximum-likelihood and Bayesian tree topologies obtained for the 3 segments were similar, only the small segment is presented. The Figure shows the Bayesian tree topology based on all RVFV small segments, with the HB29 phlebovirus from the People's Republic of China as an outgroup. Tree topologies are consistent with those generated in previous work (*7*,*11*). The reliability of the phylogenetic trees was confirmed by performing bootstrap analysis. The Kenya 1 and Kenya 2 lineages clustered together with an overall bootstrap value of 92% but with sublineage bootstrap values of 56%–100%.

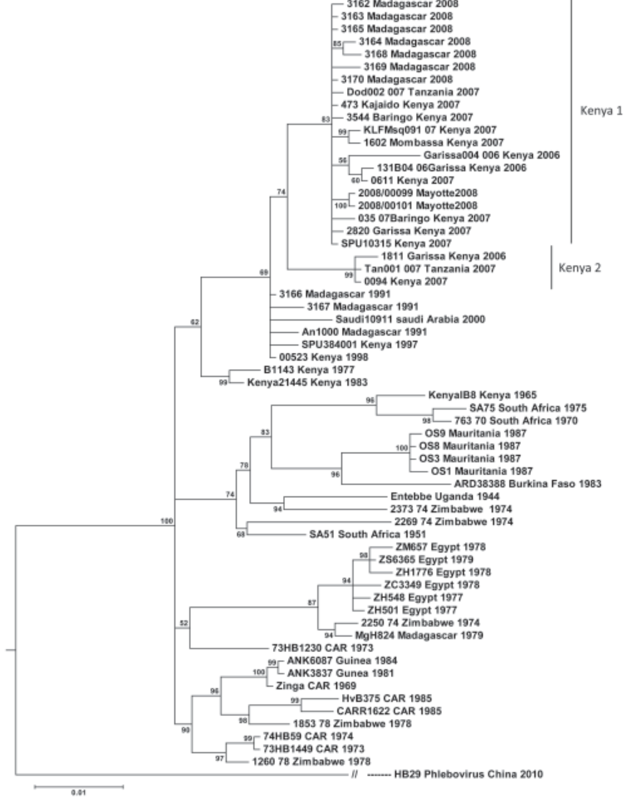


Figure. Fifty-two complete sequences of Rift Valley fever virus small genome segments aligned and analyzed by the Bayesian program (MrBayes). Scale bar indicates nucleotide substitutions per site.

## Conclusions

The work of Sissoko et al. (*9*) suggested the indigenous transmission of RVFV in humans in Mayotte. The geographic distribution of the 10 human serum samples found positive for RVFV in 2007 and 2008 was not spatially delimited. All case-patients were native to the island and resided in the following districts: Mamoudzou (3), Brandaboua (2), Dembeni (1), Sada (1), Chirongui (2), and Boueni (1). None reported travel into countries where RVF is endemic (*9*). The genomic analysis of the Mayotte isolates placed them within the 2006–2007 eastern African Kenya-1 lineage. RVF activity in Mayotte appears to be an expansion of the eastern African mainland 2007–2008 outbreak. It illustrates the risk for introduction in Mayotte or other Comorian islands of infectious agents involved in outbreaks in neighboring eastern African coastal countries, the major source being livestock importation from the African mainland or Madagascar.

The recent data published on RVFV Malagasy strains (*7*,*14*) support an epidemic cycle with introduction of the virus from outbreaks on mainland eastern Africa rather than an enzootic cycle in Madagascar. RVFV has been isolated from at least 40 species of mosquitoes in 8 genera. Recent experimental RVFV infections on African mosquito species revealed that 8 species—*Aedes palpalis* (Newstead), *Ae. mcintoshi* Huang, *Ae. circumluteolus* (Theobald), *Ae. calceatus* Edwards, *Ae. aegypti* (L), *Culex antennatus* (Becker), *Cx. pipiens* (L), and *Cx. quinquefasciatus* Say—are susceptible to infection, and that all except *Ae. calceatus, Ae. aegypti*, and *Cx. quinquefasciatus* transmitted RVFV by bite after oral exposure (*15*). In Mayotte, a preliminary study has shown that 4 species—*Ae. circumluteolus*, *Cx. antennatus*, *Cx. quinquefasciatus*, and *Ae. aegypti*—are present (T. Balenghien, V. Robert, pers. comm.).

Even if mosquito transmission might have occurred among some of the 7 reported RVF case-patients, contact with imported ruminants is the predominant means of exposure among these reported case-patients. However, further entomologic studies need to be conducted to

identify all potential vector species in the island and animal surveys need to be conducted to help detect RVF at early stages to gain a better understanding of the ecologic and climatic factors that favor RVFV dissemination. These assessments will help in the development of appropriate control measures to better predict and respond to potential RVF outbreaks.


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# Prevalence of Rift Valley Fever among Ruminants, Mayotte

**Catherine Cêtre-Sossah, Aurélie Pédarrieu, Hélène Guis, Cédric Defernez, Michèle Bouloy, Jacques Favre, Sébastien Girard, Eric Cardinale, and Emmanuel Albina**

Rift Valley fever threatens human and animal health. After a human case was confirmed in Comoros in 2007, 4 serosurveys among ruminants in Mayotte suggested that Rift Valley fever virus had been circulating at low levels since 2004, although no clinical cases occurred in animals. Entomologic and ecologic studies will help determine outbreak potential.

Rift Valley fever virus (RVFV) usually causes large, explosive epidemics among animals and humans and circulates in many African countries and the Arabian Peninsula (*1–3*). The human and veterinary medical role of this mosquito-borne virus was highlighted at the end of 2006 and early 2007, when a large epidemic/epizootic occurred in eastern Africa (*4*,*5*) and Madagascar, during 2 successive rainy seasons (*6*,*7*). More recently, South Africa and Mauritania were severely affected (*8*,*9*). This wide dissemination potential emphasizes that Rift Valley fever constitutes a threat for human and animal health on the African continent and beyond. In Mayotte in July 2007, recent RVFV infection was detected in a 12-year-old boy with a severe neuroinvasive illness. This patient had recently arrived from Grande Comore, Union of the Comoros, where RVFV circulation had been confirmed (*10–12*).

Starting in April 2008, given the proximity of Comoros and Mayotte and considering the risk for introducing RVFV by illegal animal movements, active laboratory-based surveillance for Rift Valley fever was implemented among susceptible ruminants in Mayotte. A series of 4 serosurveys was designed to clarify the epidemiologic situation. The first survey captured information about goats and cattle illegally introduced to the northern part of the island of Mayotte, the site of most illegal imports because of its proximity with the Comoros island of Anjouan (Figure 1). The second survey was a retrospective islandwide serologic survey of ruminant samples collected during 2007–2008, intended to capture a broader view of the situation. The third survey was a 4-year retrospective serosurvey of ruminant samples collected during 2004–2007, intended to increase knowledge of the history of the virus on the island. The fourth survey, a longitudinal serologic study on goat farms, assessed whether the virus was still circulating in 2008.

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## The Study

The first survey, intended to clarify the Rift Valley fever epidemiologic situation on the island, was undertaken in the M'Tsangamouji area (northern part of Mayotte). It examined samples from 29 illegally introduced goats and 79 cattle born on the island and living near the goats. Among the 29 goats, competitive IgG ELISA found IgG against RVFV in 4 goats that had been introduced illegally during November 2007–April 2008 (*13*), and IgM-capture ELISA found IgM against RVFV in 2 goats (*14*), suggesting recent infection. Among the 79 cattle, IgG against RVFV was found in 29 (37%) and IgM against RVFV was found in 3 (4%).

These data led us to conduct the second survey, a retrospective study on the whole island to define the geographic distribution of the infection and to trace back the period of introduction. This survey analyzed 301 cattle

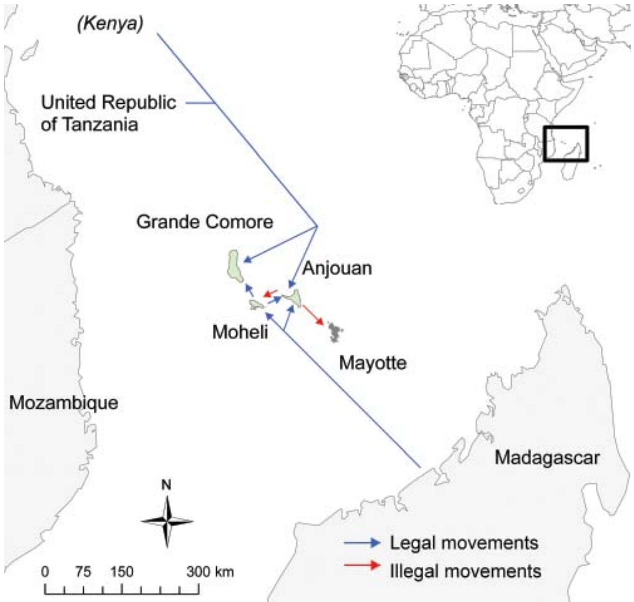


Figure 1. Potential legal and illegal movements of animals around the Comoros and Mayotte.

serum samples collected during June 2007–May 2008 on 104 farms in 17 districts. Exposure to RVFV was indicated by competitive IgG ELISA detection of RVFV-specific antibodies. Positive results were found for 32 samples from cattle in 9 districts (Table). The overall apparent RVFV seroprevalence of 10.6% (95% CI 7%–14%) was supported by the high specificity of the ELISA (*14*). The 32 positive samples came from cattle distributed all over the island (Figure 2, panel A).

Because RVFV circulation had been confirmed as early as 2007–2008 in Mayotte, a third cross-sectional and retrospective study was conducted to trace previous virus circulation. The 120–130 samples that had been collected from cattle since 2004 were randomly selected every year over a 4-year period and analyzed by IgG ELISA; results were confirmed by neutralization tests (*15*). These results helped evaluate RVFV circulation on Mayotte island before the 2007–2008 outbreak on the eastern Africa mainland. In 2004, a total of 29 of 130 cattle had IgG against RVFV; thus, seroprevalence was high (22.66%). In 2005, seroprevalence rates fell; IgG against RVFV was found in only 4 of the 130, suggesting low levels of RVFV circulation. In 2006 and 2007, seroprevalence increased; IgG against RVFV was found in 16 of 130 and 39 of 126 cattle, reaching seroprevalence rates of 12.31% and 30.95% for 2006 and 2007, respectively. Specific IgM against RVFV was not detectable during this cross-sectional and retrospective study.

Table. Rift Valley fever virus seroprevalence among cattle, Mayotte, June 2007–May 2008

| District | No. positive/no. tested | Seroprevalence, % |
|---|---|---|
| Acoua | 0/6 | 0 |
| Bandraboua | 5/31 | 16.13 |
| Bandrele | 0/6 | 0 |
| Boueni | 0/1 | 0 |
| Chiconi | 0/9 | 0 |
| Chirongui | 1/3 | 33.33 |
| Dembeni | 7/32 | 21.88 |
| Dzoumogne | 0/1 | 0 |
| Kahani | 1/5 | 20 |
| Kani Keli | 3/26 | 11.54 |
| Koungou | 0/10 | 0 |
| Mamoudzou | 0/28 | 0 |
| Mirereni | 1/2 | 50 |
| Mtsangamouji | 6/21 | 28.57 |
| Ouangani | 3/40 | 7.50 |
| Sada | 0/20 | 0 |
| Tsingoni | 5/60 | 8.33 |
| Total | 32/301 | 10.63 |

The fourth survey, intended to evaluate the recent virus dynamics in Mayotte, was a longitudinal serologic survey

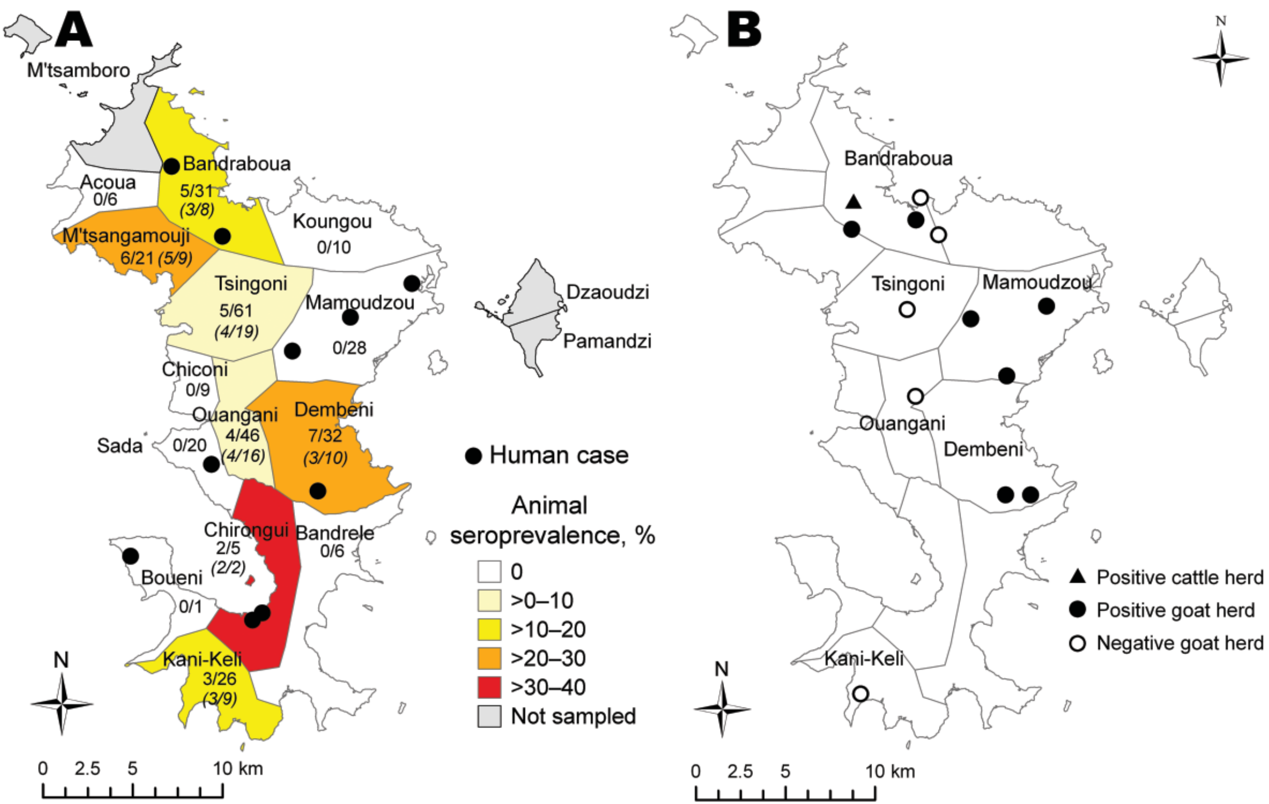


Figure 2. Rift Valley fever in Mayotte, by municipality. A) Human cases and animal and herd seroprevalence. Values under municipality names are seroprevalence by herd (no. infected herds/no. herds) and, in parentheses, by animal in infected municipalities (no. infected/total no.). B) Status of goat herds sampled for longitudinal serologic study, Mayotte, 2008–2009.

of goat farms. In June 2008, a total of 13 goat farms were selected and all 272 animals were screened for antibodies against RVFV. Of the 13 farms, 8 had seropositive animals (herd prevalence 62%, 95% CI 35%–88%) (Figure 2, panel B). Intraherd prevalence ranged from 6% to 42%. The 5 farms with seronegative goats (total 70 goats) were included in the longitudinal study. During August 2008–August 2009, the seronegative goats were sampled every 6–8 weeks. Only 1 goat, located in Bouyouni and sampled in February 2009, had seroconverted and was confirmed IgM positive for RVFV. Virus isolation attempts were unsuccessful.

## Conclusions

The 4 serologic surveys conducted in Mayotte revealed medium to high rates of RVFV prevalence all over the island. The high rates obtained with the first survey in the M'Tsangamouji area suggest that illegal animal movements from the Comoros are a likely source for RVFV introduction onto Mayotte. Results of the 4-year survey show that the virus was already present in 2004. After a low seroprevalence rate in 2005, the increased seroprevalence rates for 2006 and 2007 suggest that the virus had recirculated or had been newly introduced.

It is unclear why relatively high circulation of RVFV in Mayotte and an increased rate of seroprevalence to 22% did not result in detectable clinical cases in animals while Rift Valley fever was diagnosed for humans with brain disorders (*11*). This finding might be because the density of susceptible animals on the island was high enough to support virus circulation but too low to support waves of epidemic abortion and death. These study findings, coupled with epidemics in eastern Africa, illustrate the risk for introduction of infectious agents from the African mainland to Mayotte or other Comoros islands. Entomologic studies need to be conducted to identify all potential vector species on the island and to better understand the ecologic and climatic factors that favor RVFV dissemination. The ecologic factors in favor of Rift Valley fever outbreaks might be comparable between Mayotte, the other Comoros islands, Madagascar, and the eastern African mainland (Kenya, Tanzania, and Mozambique) but need to be looked at more closely. In Mayotte, an entomologic surveillance program is being developed to help define the distribution of potential vectors in association with virus circulation and provide better understanding of disease spread mechanisms. The role of wildlife should also be investigated. These data highlight the need for extensive studies to determine RVFV distribution and to evaluate the effect of Rift Valley fever on the susceptible livestock populations.

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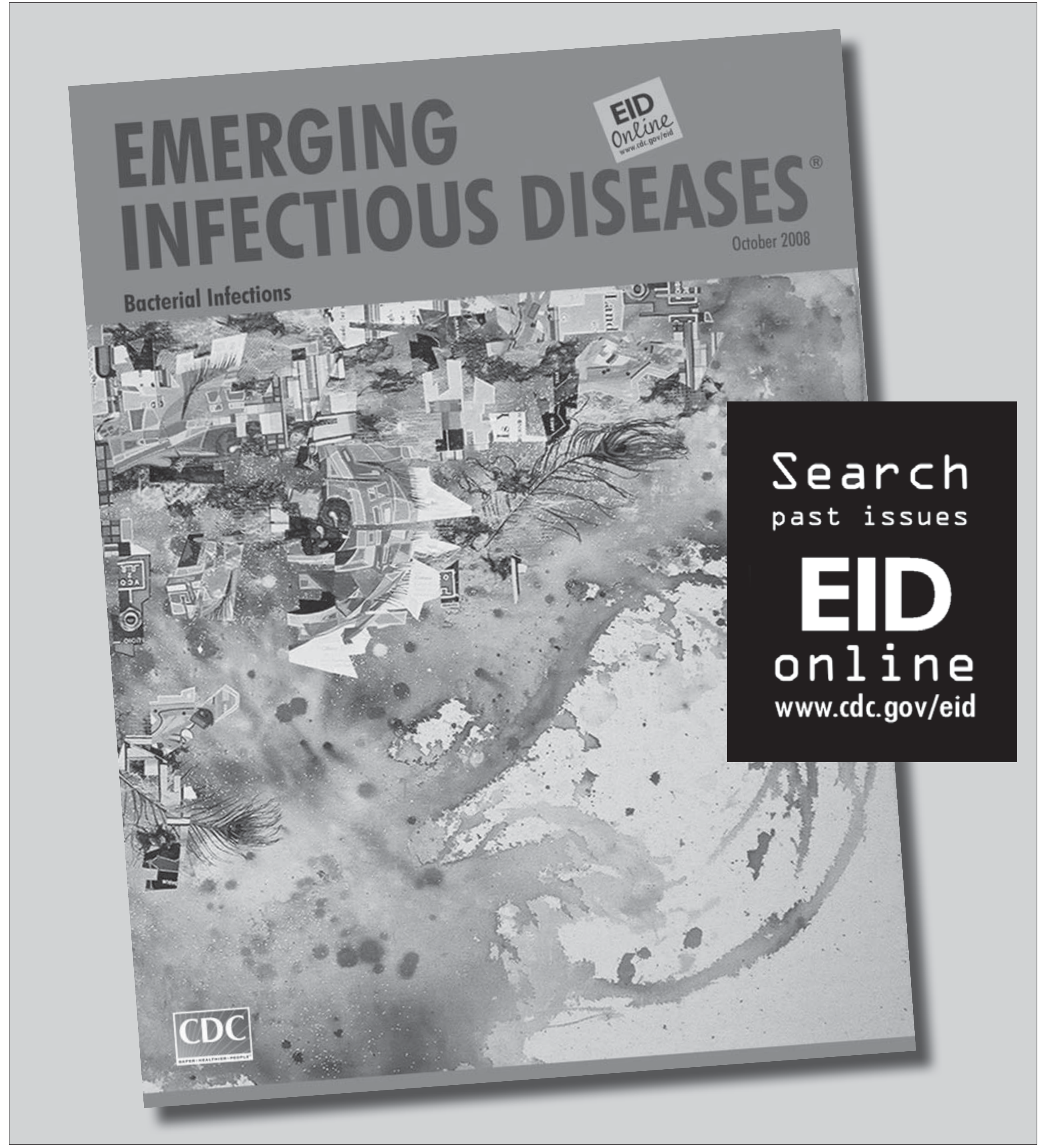

# Louping Ill in Goats, Spain, 2011

Ana Balseiro, Luis J. Royo, Claudia Pérez Martínez, Isabel G. Fernández de Mera, Úrsula Höfle, Laura Polledo, Nelson Marreros, Rosa Casais, and Juan F. García Marín

Although louping ill affects mainly sheep, a 2011 outbreak in northern Spain occurred among goats. Histopathologic lesions and molecular genetics identified a new strain of louping ill virus, 94% identical to the strain from Britain. Surveillance is needed to minimize risk to domestic and wildlife species and humans.

Louping ill is a zoonotic disease caused by a neurotropic, single-stranded, 40 to 50–nm RNA virus, which has been classified in the family *Flaviviridae*, genus *Flavivirus*. Louping ill virus belongs to a subgroup antigenically related to viruses known as the tick-borne encephalitis viruses (TBEVs) of Europe (*1*). These viruses are medically considered to be the most common flaviviruses in Europe and Asia (*2*). Among them, the TBEVs, which infect thousands of humans per year (*3*), are related to louping ill virus. Most reports of louping ill virus originate from the British Islands; knowledge with regard to the strain from Spain is limited. Because it is a tick-transmitted disease, the distribution of louping ill is closely associated with the distribution of the primary vector, the tick *Ixodes ricinus*. It mainly affects sheep and red grouse (*Lagopus lagopus scotica*), but many other species have been reported to be susceptible, including dogs, llamas, alpacas, goats, pigs, and humans (*2*).

Infection with a strain of louping ill virus from Spain was first reported for sheep in the Basque region of northern Spain in 1987. For several years during the spring, mortality rates for lambs and yearlings in infected flocks were high (*4*). The causative agent was identified as Spanish sheep encephalitis virus (SSEV) (*5*). Additional studies failed to isolate the virus from ticks (*6*) in the region where the first cases occurred, indicating that prevalence of SSEV, if present, was low. To our knowledge, no cases of encephalitis caused by a flavivirus in ungulates in Spain have been described since then.

Also to our knowledge, no cases of tick-borne flavivirus infection in humans have been reported in Spain, although 1 case of tick-borne encephalitis in a person from southwestern France was considered to have been caused by an SSEV subtype virus (*3*,*7*). Therefore, except for the 1 person in France, SSEV infections seem to be restricted to sheep and to the Basque region of northern Spain. We report suspected infection of a herd of Bermeya goats (an endangered breed of Asturian goats) with a TBEV.

## The Cases

In September 2011, a herd of 70 adult goats was purchased in southern Asturias and then moved to northern Asturias. Within 1 month, 1 goat became ill. The first sign was hindleg lameness, which quickly progressed to incoordination, fever, tremors, and bulging eyes. The goat died 2 days later, after which 17 other goats (including 2 goatlings born on the farm) showed the same signs and died over a 4-month period. Many ticks were found on these animals (≈10–15 ticks/goat), and Butox (Merck, Madrid, Spain) was applied.

Necropsy was performed on 2 adult goats; gross lesions were recorded and special attention was paid to the nervous system. Samples for histopathologic examination were taken from the brain (cerebrum, midbrain, cerebellum, and brain stem), spinal cord, liver, kidney, adrenal glands, lungs, spleen, and gastrointestinal tract. They were fixed in 10% neutral-buffered formalin, and 4-µm hematoxylin and eosin–stained sections were produced.

Samples of brain tissue were also taken for molecular analysis. Total RNA was extracted (TRIzol reagent; Gibco BRL, Grand Island, NY, USA), treated with DNase 1 (Takara Bio Inc., Kyoto, Japan), and reverse transcribed into single-stranded cDNA by using random hexamers (First Strand cDNA Synthesis Kit for reverse transcription PCR (RT-PCR) [avian myeloblastosis virus]); Roche Diagnostics, Indianapolis, IN, USA). Real-time RT-PCR primers designed to detect all viruses in the family *Flaviviridae* (*8*) were used in a conventional RT-PCR protocol, and a 231-bp amplicon was detected and sequenced (BigDye Terminator, version 3.1, Cycle Sequencing Kit protocol; Applied Biosystems, Foster City, CA, USA). To compare the phylogenetic relationships of the isolate virus with other representative TBEV strains, we constructed an unweighted pair-group method analysis tree in MEGA 5 (*9*), by using published TBEV sequences (*8*) and the Three Arch Rock Island strain of Tyuleniy virus as an outgroup.

During necropsy of the 2 goats, no gross lesions were found. No histopathologic lesions were found in any organ system except the central nervous system (Figure 1). The

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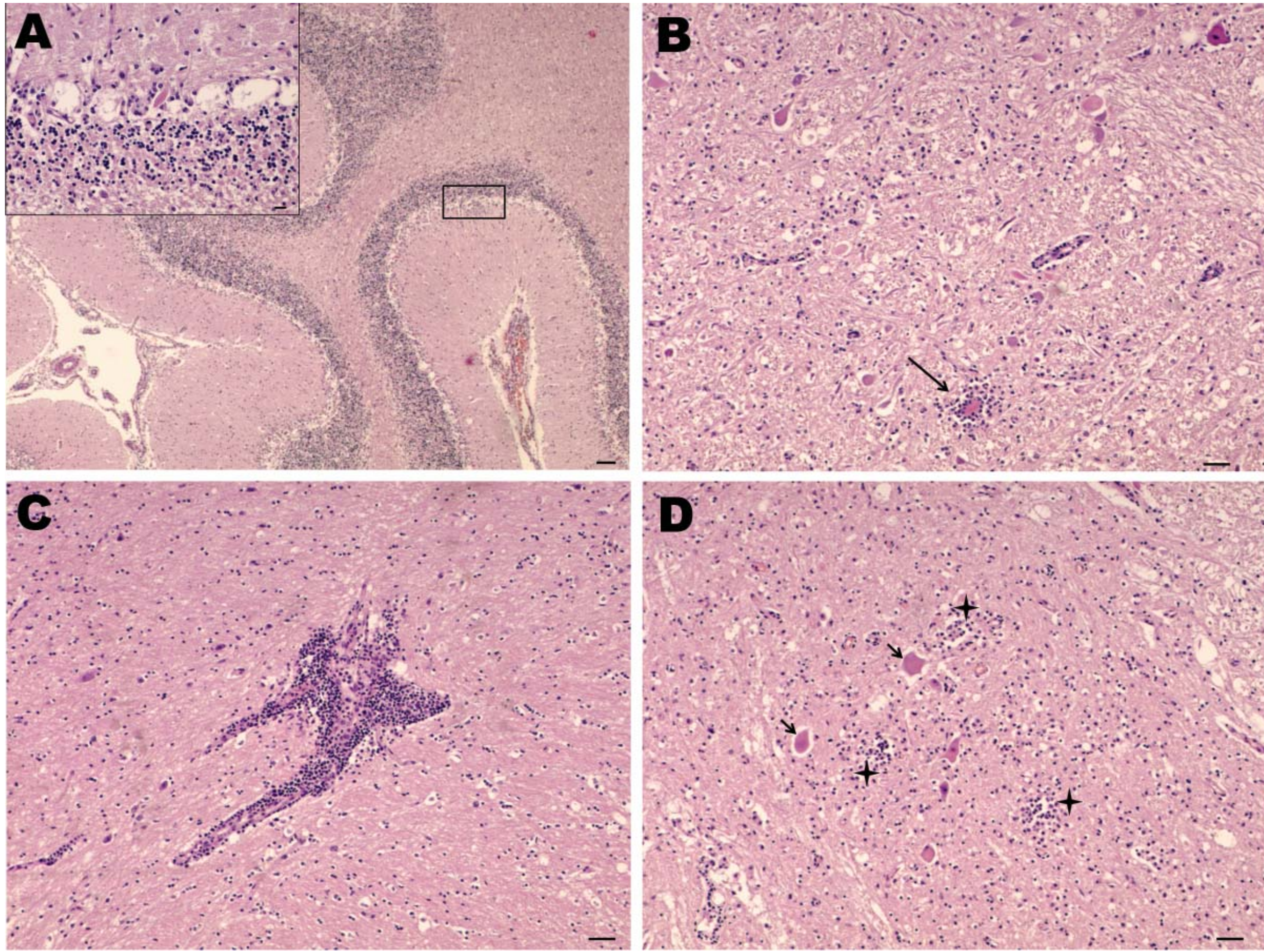


Figure 1. Nonsuppurative encephalitis in goat affected by louping ill. A) Cerebellum with necrosis of Purkinje cells. Hematoxylin and eosin (H&E) stain; scale bar = 100 μm. Inset: necrosis of Purkinje cells. H&E stain; scale bar = 20 μm. B) Midbrain. Area of neurophagia (arrow) surrounded by microglial cells. Necrosis of neurons can be also seen. H&E stain; scale bar = 50 μm. C) Lymphoid perivascular cuff in midbrain. H&E stain; scale bar = 50 μm. D) Spinal cord, gray matter. Focal microgliosis (crosses) and neurons undergoing necrosis (arrows). H&E stain; scale bar = 50 μm.

cerebellum showed necrosis of Purkinje cells and neurons. Histopathologic examination of the rest of the brain revealed a mild meningeal infiltration with widespread lymphocytic perivascular cuffs and evidence of neurophagia and gliosis, characterized by degenerating neurons surrounded by glia cells. These lesions were concentrated in the hypothalamus and midbrain and were more severe in the medulla oblongata and spinal cord. The histopathologic lesions observed in these goats were indistinguishable from those caused by louping ill virus (*10*).

Molecular genetic studies enabled identification of the virus. The sequence (Genbank accession no. JQ646028) was 94% identical to the strain from Britain (EU074000) and 93% identical to the strains from Spain (EU074016) and the Negishi virus (EU074002), thus confirming identity of the Asturian strain louping ill virus. Phylogenetic analysis, conducted by using the unweighted pair-group method analysis tree (Figure 2) shows how the new virus strain is related to the strains from Britain and Spain.

## Conclusions

Histopathologic lesions together with molecular genetic results enabled a definitive diagnosis: tick-borne encephalitis caused by an Asturian strain of louping ill virus. These cases confirm the infection in species other than sheep in Spain and the presence of the virus in areas of northern Spain other than the Basque region. An epidemiologic survey confirmed that no clinical signs were observed for the source herd in southern Asturias. However, a few years ago, another herd, located in the same area to which the affected herd described here had been moved, showed similar signs; 8 of 20 goats died. Although a diagnosis was not confirmed for this earlier outbreak, the facts suggest that the virus might have been in this area for several years.

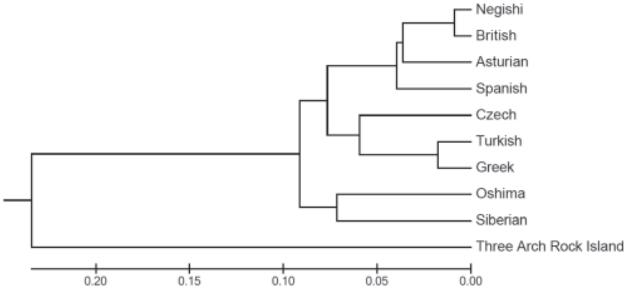


Figure 2. Phylogenetic relationships of the Asturian strain louping ill virus with representative tick-born encephalitis viruses. Phylogenetic and molecular evolutionary analyses of the virus were conducted using MEGA version 5 (*9*). Scale bar indicates branch length, proportional to the number of nucleotide substitutions. The Three Arch Rock Island virus was included as an outgroup.

The concern over finding louping ill in this area lies in its zoonotic potential. Numerous cases of human infection have been described (*11*). Humans can become infected in a variety of ways. Infections have been naturally acquired in persons who had direct contact with infected animals, for example on the farm or in laboratory settings (*11*,*12*). The virus can also be transmitted by direct mucous or respiratory pathways. Another route for infection is the consumption of milk from infected goats or products (cheese, butter, or yogurt) made from milk from infected goats (*13*). The presence of the virus in milk could represent a public health hazard if the milk is not pasteurized.

The Bermeya goat is considered to be at high risk for extinction, and many efforts have been made by breeders and the local administration of agriculture to limit the loss of genetic variability in these goats. The cases reported here provide an example of how an infectious disease can also reduce the local genetic resources. These cases, together with the case described in the Basque region, underline the need for a specific surveillance plan in northern Spain that focuses on ticks, wildlife species, and livestock. This plan will be crucial for determining the actual effects of louping ill on hunting, animal breeding, and human health.

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# Accuracy of ICD-10 Codes for Surveillance of *Clostridium difficile* Infections, France

**Gabrielle Jones, Namik Taright, Pierre Yves Boelle, Jeanne Marty, Valérie Lalande, Catherine Eckert, and Frédéric Barbut**

The sensitivity and specificity of surveillance for *Clostridium difficile* infections according to International Classification of Diseases, 10th revision, codes were compared with laboratory results as standard. Sensitivity was 35.6%; specificity was 99.9%. Concordance between the 2 methods was moderate. Surveillance based on ICD-10 codes underestimated the rate based on laboratory results.

*Clostridium difficile* causes 15%–25% of diarrhea after antimicrobial drug therapy and is the leading cause of nosocomial diarrhea in adults (*1*). Studies in the United States, Canada, and Europe have documented the increased rate and severity of *C. difficile* infections highlighting the need for efficient and accurate methods of surveillance (*2*–*7*). The use of International Classification of Diseases (ICD) codes for surveillance of *C. difficile* infections has been studied in the United States and in Singapore and showed discordant results (*8*–*12*). Our objective was to compare the sensitivity and specificity of surveillance for *C. difficile* infections on the basis of ICD, 10th revision (ICD-10), codes with surveillance based on laboratory results.

## The Study

The study was conducted at Saint-Antoine Hospital, a 750-bed university-affiliated public hospital in Paris, France. The study population comprised all patients hospitalized during January 1, 2000–December 31, 2010. *C. difficile* testing was performed only on unformed fecal samples of patients clinically suspected to have *C. difficile* infection. Laboratory diagnosis of *C. difficile* infection did not vary during the study period and was based on the stool cytotoxicity assay coupled with the toxigenic culture. A bacteriologic case of *C. difficile* infection was defined as a positive cytotoxicity assay result and/or a positive toxigenic culture.

Data were collected retrospectively from the electronic discharge summaries (French medico-administrative database) and from the hospital microbiology laboratory. All patients with a positive laboratory result for *C. difficile* (Bact+) and/or the ICD-10 discharge code for *C. difficile* infection, A04.7, as principal or associated diagnosis (ICD10+), were identified. For patients with multiple laboratory results during the same hospitalization, we used only the initial result.

We classified cases as concordant (Bact+/ICD10+) or discordant (Bact+/ICD10– or Bact–/ICD10+). Bact+/ICD10– discordant cases were compared with concordant cases to identify factors predictive of missing codes. Medical records were reviewed for Bact–/ICD10+ case-patients.

Statistical analysis included κ, $\chi^2$, and the Mann-Whitney U test. We used the Spearman test to measure the correlation between the 2 methods for yearly incidence of *C. difficile* infection. Data were analyzed with Epi Info version 6.01 (Centers for Disease Control and Prevention, Atlanta, GA, USA), GraphPad Prism version 4.03 (GraphPad Software, Inc., La Jolla, CA, USA) and R version 2.0 (R Foundation for Statistical Computing, Vienna, Austria).

During 2000–2010, of 317,040 hospitalizations, laboratory results and/or the ICD-10 code for *C. difficile* infection were positive for 698 (Figure 1). Sensitivity of the ICD-10 code, with laboratory results as the standard, was 35.6% (95% CI 31.9%–39.5%), and specificity was 99.9% (95% CI 99.9%–100.0%). The positive and negative predictive values were 79.2% (95% CI 73.9%–83.7%) and 99.9% (95% CI 99.8%–99.9%), respectively (Table). The sensitivity of ICD-10 codes varied among hospital wards. For wards with >50 cases of *C. difficile* infections during 2000–2010, sensitivity ranged from 14% to 71.6%. Average sensitivity increased from 26% for 2000–2005 to 39% for 2006–2010 ($p = 0.02$). Overall, concordance between the 2 methods was moderate ($\kappa = 0.49$, $p<0.0001$).

The incidence of *C. difficile* infection determined by ICD-10 codes underestimated the incidence determined by laboratory results. The relationship between methods for yearly incidence during the 11-year period was strong (Spearman correlation coefficient $r = 0.95$, 95% CI 0.81–0.98, $p<0.0001$). The rate of *C. difficile* infection by ICD-10 codes and laboratory results increased during 2000–2010 (Figure 2). The incidence of *C. difficile* infection also increased across all age groups. During 2000–2010, incidence increased by a factor of 3.3 for patients 15–44 years of age, by 2.9 for patients 45–64 years of age, and by 4.2 for patients ≥65 years of age.

Concordant cases (Bact+/ICD10+) and discordant cases (Bact+/ICD10–) did not differ significantly by

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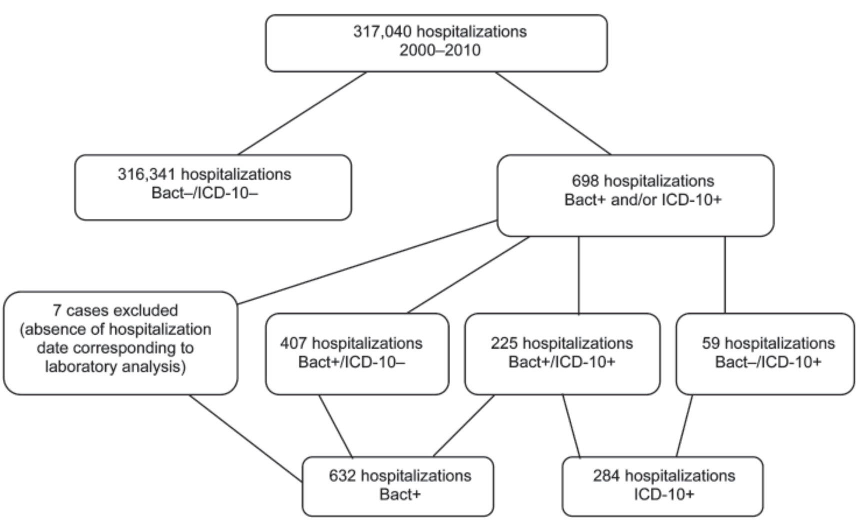


Figure 1. Flowchart of *Clostridium difficile* infections case classifications for patients admitted to Saint-Antoine Hospital, Paris, France, 2000–2010. Bact+, positive laboratory result for *C. difficile*; ICD10+, International Classification of Diseases, 10th Revision, discharge code for *C. difficile* infection, A04.7, as principal or associated diagnosis.

mean age, sex, or proportion of patients ≥65 years of age. Diagnosis by positive toxigenic culture (with negative stool cytotoxicity assay result) was predictive for missing ICD-10 codes ($\chi^2$ = 19.22, p<0.0001), as was sample collection within 48 hours before discharge ($\chi^2$ = 16.57, p<0.0001). Patients with concordant results were more likely than patients with discordant results to have sample collection within 48 hours after admission ($\chi^2$ = 23.7, p<0.0001).

Review of medical records was possible for 34 (58%) of 59 discordant cases Bact–/ICD10+. Potential explanations for coding in the absence of a positive laboratory result included diagnosis outside the hospital (8 cases), positive result for a nontoxigenic strain of *C. difficile* (7 cases), diagnosis by endoscopy (6 cases), strong clinical suspicion of disease in patients with a history of *C. difficile* infection but no positive laboratory result (5 cases), and initial positive result subsequently corrected to a negative result by the laboratory (2 cases). No explanation could be found for the ICD-10 code in 6 cases: 5 had a negative laboratory result for *C. difficile* in the medical record, and 1 had no record of clinical suspicion or fecal sample collection.

## Conclusions

This study covers an 11-year period and provides a large study population and more comprehensive analysis of the performance of ICD-10 codes. Our results indicate that surveillance for *C. difficile* infections based on ICD-10 codes underestimates the rate of *C. difficile* infections based on microbiological findings at Saint-Antoine Hospital. Even though trends in *C. difficile* infections incidence for the 2 methods correlated strongly, concordance was moderate.

Table. Sensitivity, specificity, and positive and negative predictive values of ICD-10 codes for *Clostridium difficile* infection, Saint-Antoine Hospital, Paris, France, 2000–2010*

| Classification | Bact+ | Bact– | Total |
|---|---|---|---|
| ICD-10+ | 225 | 59 | 284 |
| ICD-10– | 407 | 316,342 | 316,749 |
| Total | 632 | 316,401 | 317,033 |

*Sensitivity 35.6%, specificity 99.9%, positive predictive value 79.2%, and negative predictive value 99.9%. Bact+, positive result for *C. difficile*; Bact–, negative result for *C. difficle*; ICD-10, International Classification of Diseases, 10th Revision.

The sensitivity of ICD-10 codes in this study is inferior to values previously reported in the United States (71%–78%) and in Singapore (49.6%) (*8*–*11*). Poor sensitivity and variability among wards could be attributed to differences in awareness by health care professionals of *C. difficile* infections and to differences in coding practices. At Saint-Antoine Hospital, coding is performed by physicians with limited training, not by trained medical coders. Therefore, the quality of coding can vary from 1 physician to another and among wards. In addition,, differences in sensitivity could be explained by changes in hospital financing. As of 2006, funding for hospitals in France has been connected to coding through Activity Based Payment (*13*). Comparison of average sensitivity before and after 2006 showed an overall increase, indicating that coding practices might improve with time as hospitals adapt to this system.

Our finding that sample collection within 48 hours before hospital discharge was predictive of missing ICD-10 codes is consistent with findings from previous studies and suggests that results obtained after patient discharge are less frequently coded (*8*,*9*,*12*). Diagnosis by toxigenic culture was also significantly associated with missing ICD-10 codes. The toxigenic culture is a long test requiring up to several days for results. At Saint-Antoine Hospital, results are provided at each step of analysis (stool cytotoxicity assay in 24 h, culture in 48 h, toxigenic culture within 5 d), which might introduce misinterpretation of preliminary results before the final comprehensive result.

Analysis of medical records for patients coded for *C. difficile* infections but lacking a positive laboratory

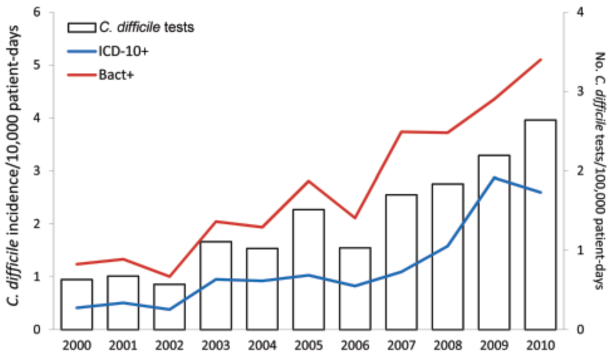


Figure 2. Incidence of *Clostridium difficile* infections by surveillance method and number of *Clostridium difficile* tests, Saint-Antoine Hospital, Paris, France, 2000–2010. Bact+, positive laboratory result for *C. difficile*; ICD10+, International Classification of Diseases, 10th Revision, discharge code for *C. difficile* infection, A04.7, as principal or associated diagnosis.

result suggested several potential explanations for coding. Diagnoses made outside the hospital and those made by endoscopy are coded, indicating that cases diagnosed by methods other than in-hospital laboratory testing are captured by ICD-10 codes.

This study was limited to a single institution, and our findings might not necessarily apply to other institutions in France. The sensitivity of ICD-10 codes can be highly variable, and this method should be validated in different health care settings before being used for surveillance.

Use of ICD-10 codes underestimates the incidence of *C. difficile* infections compared with microbiological data. However, it may be an effective indicator for monitoring general trends in the rate of *C. difficile* infection.

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# Molecular Epidemiology of Laguna Negra Virus, Mato Grosso State, Brazil

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We associated Laguna Negra virus with hantavirus pulmonary syndrome in Mato Grosso State, Brazil, and a previously unidentified potential host, the *Calomys callidus* rodent. Genetic testing revealed homologous sequencing in specimens from 20 humans and 8 mice. Further epidemiologic studies may lead to control of HPS in Mato Grosso State.

Hantavirus pulmonary syndrome (HPS) is a manifestation of an emerging zoonosis caused by New World viruses of the family *Bunyaviridae*, genus *Hantavirus*. Hantavirus is transmitted to humans by inhalation of aerosols of excreta from infected rodents of the subfamily *Sigmodontinae* (Rodentia, Cricetidae) (*1*,*2*). HPS was initially reported during an epidemic of severe respiratory disease that occurred in the southwestern United States in 1993 (*1*). HPS was subsequently identified in Brazil and other Latin American countries, which facilitated the recognition of new hantavirus species such as Laguna Negra virus (LNV), Andes virus, Choclo virus, Juquitiba virus, Araraquara virus, Castelo dos Sonhos virus, Anajatuba virus, as well as several other viruses detected in wild rodents which are not associated with HPS (*3*–*9*). Like particles of other bunyaviruses, hantavirus particles are spherical or pleomorphic and measure 80–120 nm in diameter; their genome comprises 3 RNA segments, and the small RNA fragment is used to characterize the nucleoprotein (N) gene and the hantavirus species (*2*).

During 1993–2009, a total of 1,246 cases of HPS were reported in Brazil; the state of Mato Grosso reported the fourth highest case count, diagnosed mainly in the municipalities of Tangará da Serra and Campo Novo do Parecis. However, the circulating hantavirus species and its host remained unknown, and identification of these factors were the main objectives of this study.

## The Study

Mato Grosso comprises 903,357.9 km$^2$ and has an estimated population of 2,803,274 inhabitants living in 141 municipalities. Nineteen municipalities have reported cases of HPS, mainly near Brazil's BR-364 highway, located between the north and southwestern sections of the state. The climate is equatorial subhumid, with an annual rainfall of 1,700 mm, and temperature range 24°–40°C; the landscape consists of savannah (*Cerrado*) and pre-Amazon rainforest. The economic activities are agricultural production and ecologic tourism.

HPS was diagnosed in 24 persons who were IgM positive for LNV during 2001–2006 in the municipalities of Barra do Bugres (n = 1), Campo Novo do Parecis (n = 13), Diamantino (n = 3), Nova Olímpia (n = 1), Santo Afonso (n = 1), São José do Rio Claro (n = 1), and Tangará da Serra (n = 4) (Figure 1). Detailed information of patient

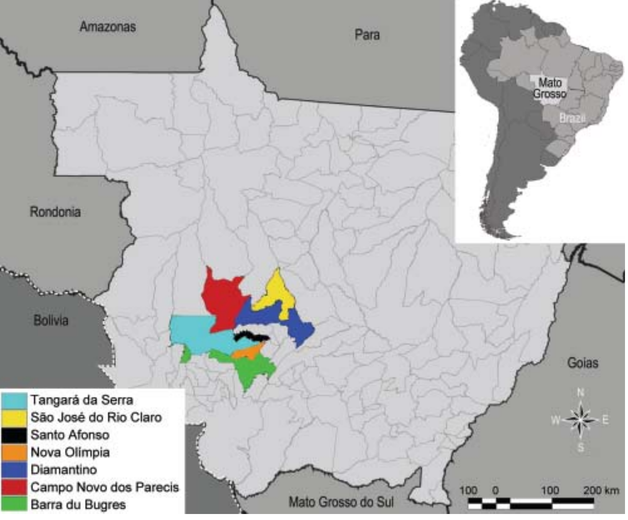


Figure 1. State of Mato Grosso, Brazil, indicating municipalities where hanta pulmonary syndrome cases occurred.

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samples submitted for nucleotide sequencing is provided in the Table.

During a 2001 ecological–epidemiologic study conducted in the municipalities of Tangará da Serra and Campo Novo do Parecis, researchers obtained blood and viscera samples from wild rodents (10). The researchers followed Brazilian Institute for the Environment and Renewable Natural Resources guidelines for the capture and handling of rodents and using biosafety level 3 protocols. The samples were tested for hantavirus; animals with positive test results were identified taxonomically by morphometry and molecular analysis of mitochondrial DNA (cytochrome b gene) (*10*,*11*).

For hantavirus detection, we conducted reverse transcription PCR to synthesize complementary DNA with generic hantavirus primers as described (*1*,*2*). We obtained N gene partial nucleotide sequences by using the Sanger method with the same primers (*3*,*4*,*6*,*9*). At least 3 amplicons per sample were sequenced in both directions to improve coverage and confidence for results. The obtained sequences were aligned with other hantavirus sequences available at the GenBank database (www.ncbi.nlm.nih.gov) with ClustalW software in BioEdit version 7.1.3 (www.mbio.ncsu.edu/BioEdit/bioedit.html). We implemented the maximum-likelihood and Bayesian methods by using PHYML (www.atgc-montpellier.fr/phyml/versions.php) and Mr. Bayes version 3.2 (http://mrbayes.scs.fsu.edu) software, respectively, for phylogenetic reconstructions. We used Modeltest version 3.7 (http://gel.ahabs.wisc.edu/mauve) to determine the best nucleotide substitution model. We analyzed 2 million replicates, with the sample fixed at every 1,000 trees generated, and used TRACER (www.evolve.zoo.ox.ac.uk) to determine whether the Bayesian analysis reached appropriate convergence (*3*,*6*,*9*,*12*).

We obtained amplicons from 20 of the 24 samples from persons with HPS and partial sequence of the N gene (≈434 bp) from 16 of the 24 samples from patients who were symptomatic at the time of sampling. During the ecologic study, 126 rodents were captured: 68 (53.9%) commensal synanthropic species, 49 (38.8%) wild rodents [*Calomys callidus* (n = 46), *Proechimys* sp. (n = 1), and *Necromys lasiurus* (n = 2)], and 9 (7.1%) unidentified species. IgG was detected in 8 (17.4%) *C. callidus* rodents (2 captured in Campo Novo do Parecis, 6 in Tangará da Serra). Amplicons were produced in lung/heart samples from 7 of the 8 IgG-positive rodents; 3 of those were selected for nucleotide sequencing of the N gene (Table).

All strains recovered from human and *C. callidus* rodent specimens were related and formed a monophylogenetic cluster with the LNV (GenBank accession no. AF005727), with a mean genetic divergence of 4.8%. These strains were included in subclade II, which comprises Anajatuba,

Table. Characteristics of human patients and *Calomys callidus* rodents with positive serologic results for hantavirus and partial nucleotide sequence of gene N, Mato Grosso, Brazil*

| ID no. | Age, y/sex | Sample | Date of sample collection | Outcome | GenBank accession no. | Municipality |
|---|---|---|---|---|---|---|
| Humans | | | | | | |
| H 678213 | 38/M | Serum | 2005 Mar 10 | Cure | JQ775513 | Barra do Bugres (15°4′21″S; 57°10′52″W) |
| H 650736 | 33/M | Serum | 2001 Dec 19 | Death | JQ775504 | Campo Novo do Parecis (13°40′31″S; 57°53′31″W) |
| H 660462 | 23/F | Serum | 2002 Jul 8 | Cure | JQ775506 | |
| H 671696 | 20/F | Serum | 2003 Aug 23 | Death | JQ775505 | |
| H 682807 | 20/M | Serum | 2004 Aug 22 | Death | JQ775507 | |
| H 695689 | 27/F | Serum | 2005 Aug 22 | Cure | JQ775508 | |
| H 696558 | 22/M | Blood | 2005 | Cure | JQ775512 | |
| H 711891 | 42/M | Serum | 2006 Aug 17 | Death | JQ775503 | |
| H 657848 | 42/M | Serum | 2002 May 14 | Cure | JQ775517 | Diamantino (14°24′31″S; 56°26′46″W) |
| H 706738 | 13/F | Serum | 2006 May 22 | Cure | JQ775516 | |
| H 712518 | 33/M | Serum | 2006 Aug 29 | Cure | JQ775518 | |
| H 653486 | 13/F | Serum | 2002 Feb 22 | Cure | JQ775514 | Nova Olímpia (14°47′50″S; 57°17′17″W) |
| H 695325 | 24/M | Serum | 2005 Nov 11 | Cure | JQ775515 | Santo Afonso (14°29′51″S; 7°0′7″W) |
| H 713175 | 17/M | Serum | 2006 Sep 26 | Cure | JQ775509 | São José do Rio Claro (13°26′48″S; 56°43′17″W) |
| H 651686 | 31/M | Serum | 2002 Jan 22 | Cure | JQ775511 | Tangará da Serra (14°37′10″S; 57°29′9″W) |
| H 710031 | 7/F | Serum | 2006 Jul 18 | Death | JQ775510 | |
| Rodents | | | | | | |
| AN 650204 | NA | Lung | NA | NA | JQ775500 | Campo Novo do Parecis |
| AN 650228 | NA | Lung | NA | NA | JQ775502 | |
| AN 649993 | NA | Heart | NA | NA | JQ775501 | Tangará da Serra |

*Source: Secretaria de Vigilância Epidemiológica do Estado do Mato Grosso and Instituto Evandro Chagas, Secretaria de Vigilância em Saúde), and Ministério da Saúde. NA, data not available.

Rio Mamore, Rio Mearim, and Alto Paraguay viruses (Figure 2). The genetic distance between strains recovered from rodents and humans was 5.5%, whereas the genetic distance between the human strains was 6.8%. Analysis of homology showed no difference between the partial amino acid sequences of human and rodent strains and LNV (100% homology). The homology of nucleotide sequences between the LNV strains was 89.9%–93.4% (online Appendix Table, wwwnc.cdc.gov/EID/article/18/6/11-0948-TA1.htm). Most changes were silent mutations in the nucleotide sequences, indicated by the genetic divergence between LNV strains (Δdiv = 0.2%–9.8%).

LNV was initially confirmed in 1997 by serologic testing of a patient with HPS who died. The patient lived in Santiago, Chile, but was probably infected in Santa Cruz, Bolivia (*13*). In 1999, molecular analysis of the small N gene and medium Gn and Gc gene segments of the hantavirus genome in samples from HPS patients from Bolivia, western Paraguay, and Chile facilitated the genetic characterization of LNV and its association with the small vesper mouse *Calomys laucha*, which is considered the primary host of LNV. Subsequent studies in Argentina have also demonstrated the circulation of LNV in patients with HPS and in the large vesper mouse *Calomys callosus* (*4*,*9*,*13*–*15*).

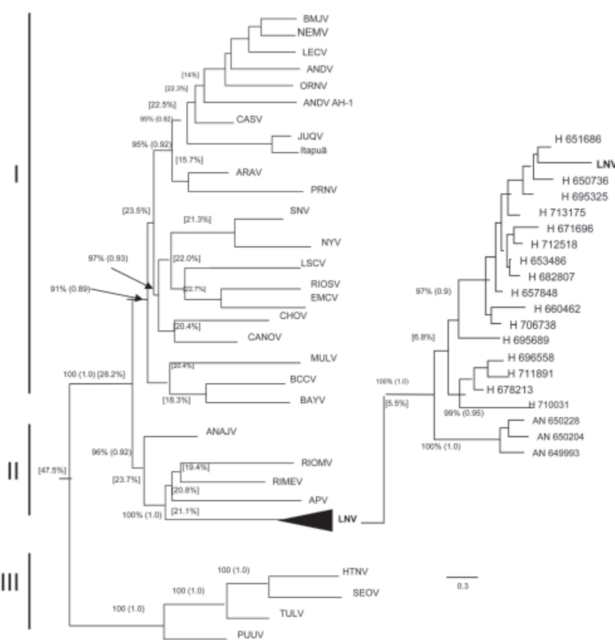


Figure 2. Phylogenetic comparison of the partial nucleotide sequences of nucleoprotein (N) gene of the small (S) RNA segment of different hantavirus strains from the Old World and New World by using the maximum-likelihood method and Bayesian analysis, with detail of the phylogenetic relationship between LNV strains isolated from humans and rodents in the state of Mato Grosso, Brazil. Bootstrap and Bayesian values shown for each respective knot. The arrows indicate the exact (within parentheses) are position of the values. Numbers within brackets correspond to the divergence between groups. Scale bar indicates 30% divergence of nucleotide sequences. APV, Alto Paraguay; ANAJV, Anajatuba, ANDV, Andes; BMJV, Andes Bermejo, NEMV, Andes Neembuco, LECV, Andes Lechiguanas; ORNV, Andes Oran; ARAV, Araraquara; BAYV, Bayou; BCCV, Black Creek Canal; CANOV, Cano Delgadito; CASV, Castelo dos Sonhos; CHOV, Choclo; EMCV, El Moro Canyon; HTNV, Hantaan; JUQV; Juquitiba-Araucaria; LNV, Laguna Negra; LSCV, Limestone Canyon; MULV, Muleshoe; NYV, New York; PRNV, Pergamino; PUUV, Puumala; RIOMV, Rio Mamoré; RIMEV, Rio Mearim; RIOSV, Rio Segundo; SEOV, Seoul; SNV, Sin Nombre; TULV, Tula.

## Conclusions

Our phylogenetic analysis of partial sequences of the N gene showed LNV as the cause of HPS, and the possible association of the organism with *C. callidus* rodents in western Brazil. These findings highlight the intense circulation of LNV in Matto Grosso municipalities located near the BR-364 highway. The vegetation and the equatorial climate of the area provide an excellent microenvironment for the maintenance of *C. callidus* rodents, as do areas in Bolivia Paraguay, and northern Argentina, where HPS caused by LNV has been reported (*4*,*9*,*13*–*15*).

The high nucleotide and amino acid homology between strains recovered from humans and the *C. callidus* rodent in Matto Grosso and the LNV prototype detected in Paraguay and Argentina suggest that LNV was transmitted by the rodent host *C. callidus* and led to the HPS cases that occurred in the vicinity of the highway BR-364 in southwestern Matto Grosso. No correlation was observed between the human LNV strains and year, geographic distribution, or between the severity of disease and the genetic diversity of LNV found in Brazil. The genetic data obtained in this study provide a better understanding of the molecular characterization of LNV and its association with HPS in southwestern Matto Grosso. Finally, on the basis of the phylogenetic analysis, the rodent species *C. callidus* is suggested as a potential reservoir for LNV. Further analyses of complete genome data are needed to confirm this result and to assess whether the *C. callidus* rodent is the sole carrier of of LNV in Matto Grosso.

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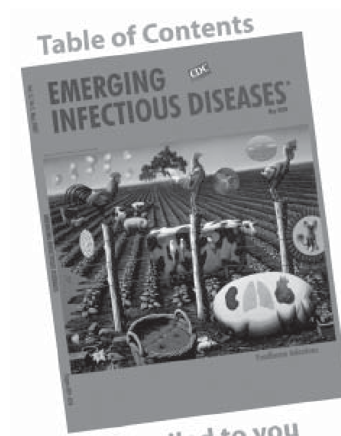


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# Avian Influenza A (H5N1) Virus Antibodies in Poultry Cullers, South Korea, 2003–2004

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Transmission of influenza (H5N1) virus from birds to humans is a serious public health threat. In South Korea, serologic investigation among 2,512 poultry workers exposed during December 2003–March 2004 to poultry with confirmed or suspected influenza (H5N1) virus infection found antibodies in 9. Frequency of bird-to-human transmission was low.

The highly pathogenic avian influenza (H5N1) virus has posed a serious public health threat since 1997, when the first transmission of the virus from birds to humans was reported in Hong Kong (*1*,*2*). In South Korea, during December 2003–March 2004, this virus caused 19 outbreaks in 7 provinces (10 outbreaks on chicken farms and 9 on duck farms), which prompted a massive mobilization to cull birds and contain the outbreak (*3*). Vaccination of poultry against influenza (H5N1) virus was legally prohibited, and a stamping out policy was considered as a control option. Culling of ≈5 million birds was conducted on all farms with infected poultry and all poultry farms within a 3 km–radius protection zone.

All persons who participated in the culling operations were equipped with World Health Organization (WHO)–recommended personal protective equipment (PPE) (*4*). To prevent the possibility of mixed infection with human and avian influenza viruses, previously nonvaccinated participants were vaccinated with a seasonal influenza vaccine and given oseltamivir as an additional prophylactic measure.

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During the outbreaks, 142 respiratory specimens were collected from persons who had influenza-like illness and tested by reverse transcription PCR selective for the matrix and hemagglutinin (H) 5 genes and by virus isolation in cell culture; however, no influenza (H5N1) virus was detected (Korea Centers for Disease Control and Prevention [CDC], unpub. data).

The definition of a case of influenza-like illness was sudden onset of fever (≥38°C) with cough or sore throat. According to previous serosurveys and outbreak investigations, influenza (H5N1) virus is poorly transmitted from birds to humans (*5*–*8*). To trace the frequency of transmission of the influenza (H5N1) virus to persons who had been exposed to the confirmed or suspected virus-infected poultry, a serosurvey was conducted by the Korea CDC.

## The Study

A serologic investigation was performed among 2,512 persons who worked on poultry farms or culled birds during the 2003–2004 outbreaks in South Korea. Their use of PPE, receipt of oseltamivir, and exposure to birds with confirmed influenza (H5N1) is unclear. Poultry culling was conducted during December 12, 2003–March 21, 2004. Blood was collected from cullers on the day of culling completion in each region. Convalescent-phase blood samples were collected at least 4 weeks later. Written agreement was provided before blood was collected from cullers, other poultry workers, and their household members. This study was reviewed and approved by the ethics committee of Korea CDC.

WHO-recommended laboratory tests and case definitions were used for serologic diagnosis of influenza (H5N1) virus infection in the cohort (*9*–*11*). Before this study, the laboratory staff of Korea CDC received 4 weeks of training at the US CDC on serologic testing for influenza (H5N1) virus. All experiments with live viruses were conducted at the biosafety level-3 facility of Korea CDC, and all serologic testing at the US CDC was conducted under biosafety level-3 containment including enhancements required by the US Department of Agriculture and the Select Agent Program.

All serum samples were tested for antibodies against influenza (H5N1) virus by microneutralization (MN) assay; results were considered to be positive if titers against H5 were ≥80 according to at least 2 independent assays. As recommended by WHO, samples that were antibody-positive by MN underwent confirmatory testing by hemagglutination inhibition assay with horse erythrocytes or by H5-specific Western blot analysis (*9*,*10*).

During the 2003–2004 outbreaks, 4,108 serum samples were collected from 2,820 persons. However, ≈16% of the samples showed cytotoxicity (all cells were detached on the

Table 1. Demographic characteristics of 2,512 bird cullers exposed during December 2003–March 2004 to poultry with confirmed or suspected influenza (H5N1) virus infection, South Korea

| Characteristic | No. (%) cases | No. (%) positive |
|---|---|---|
| Age group, y, n = 2,055* | | |
| <20 | 74 (3.6) | 0 |
| 20–29 | 783 (38.1) | 5 (0.58) |
| 30–39 | 365 (17.8) | 1 (0.27) |
| 40–49 | 427 (20.8) | 3 (0.70) |
| 50–59 | 249 (12.1) | 0 |
| >59 | 157 (7.6) | 0 |
| Sex, n = 2,512 | | |
| M | 2,112 (84.1) | 9 (0.43) |
| F | 400 (15.9) | 0 |
| Type of work, n = 1,573 | | |
| Poultry farm worker† | 176 (11.2) | 0 |
| Culler | 1,327 (84.3) | 9 (0.68) |
| Other‡ | 70 (4.5) | 0 |

*No. persons whose epidemiologic history was available.
†Includes farm workers and their household members.
‡Includes epidemiologists, public health officials, and media reporters.

96-well microplates after fixation with acetone) on MDCK cells during MN assay. In total, 3,448 samples from 2,512 persons were analyzed, among which paired samples were available from 936 (37%) and a single sample was available from 1,576 (63%). The median age of the participants was 36.0 years (range 3–96 years), and 2,112 (84.1%) were male. Among those for whom epidemiologic data were available, 1,327 (84.3%) were cullers and 176 (11.2%) were farm workers or their household members (Table 1). Cullers included local government workers, soldiers, animal husbandrymen, and civilians. The culling periods were 1–13 days, and the average was 5.4 days.

Among the 2,512 persons, MN assay results were confirmed positive for 9. The US CDC confirmed positive results in a single sample for 4 persons; the Korea CDC confirmed positive results in paired samples for 5 others. Among the 9 persons with positive MN results, only 2 had positive results according to horse hemagglutination inhibition assay; however, all 9 had clear reactivity to H5 proteins on Western blot analysis and were confirmed positive according to WHO criteria (Table 2). All those with influenza (H5N1)–positive results were male, median age was 32.5 years (range 22–48 years), and all had participated in culling during the outbreaks (Table 2). None of the other poultry farm workers had seropositive results.

## Conclusions

By identifying only 9 seropositive cases among 2,512 persons, we determined that the risk for poultry-to-human transmission of the influenza (H5N1) virus is small. Other studies have also shown low frequencies of poultry-to-human (H5N1) virus transmission. In provinces in Thailand, blood samples were collected from 322 poultry farmers 6 months after confirmation of influenza (H5N1) virus outbreaks; all antibody titers were negative (*5*). Two studies of villagers in Cambodia who had frequent and direct contact with poultry with confirmed and suspected influenza (H5N1) virus infection found low frequency of virus transmission from poultry to humans (*6*,*7*). Similarly, a study in Nigeria also found negative results for antibodies against influenza (H5N1) virus among 295 poultry workers (*8*).

Table 2. Characteristics and serologic results of persons with influenza (H5N1) antibody–positive serum samples, South Korea, 2003–2004*

| | | | Serologic results‡ | | |
|---|---|---|---|---|---|
| Participant no.† | Age, y | Occupation | Neutralizing antibody titer | Horse HI titer | Western blot§ |
| 1 | 48 | Fireman | <20/80 | <10/80 | –/+ |
| 2 | 23 | Soldier | <20/80 | <10/160 | –/+ |
| 3 | 23 | Soldier | 20/640 | <10/40 | +/+ |
| 4 | 23 | Soldier | 20/160 | <10/<10 | +/+ |
| 5 | 25 | Soldier | 20/160 | <10/<10 | ±/+ |
| 6 | 48 | Provincial officer | 160 | <10 | + |
| 7 | 22 | Soldier | 80 | <10 | + |
| 8 | 36 | Provincial officer | 80 | 160 | + |
| 9 | 45 | Animal husbandryman | 80 | 80 | + |

*All 9 persons were male, worked as cullers, and had no signs or symptoms of respiratory illness. HI, hemagglutination inhibition assay; +, positive; –, negative; ±, equivocal.
†Cases 1–5 were confirmed at the Korea Centers for Disease Control and Prevention with paired samples and 6–9 were confirmed at the US Centers for Disease Control and Prevention with a single sample.
‡Results for participants 1–5 were obtained from 2 samples and for 6–9 from 1 sample.
§Western blot analyses were performed by using purified baculovirus-expressed influenza A/Vietnam/1203/2004 virus hemagglutinin as antigen.

Because our study was conducted as a public health response, it has the following limitations. We were unable to systematically assess symptoms, extent of exposure, compliance with PPE use, and taking of oseltamivir. It is not clear if the participants were exposed to birds with confirmed or suspected influenza (H5N1) virus or whether they wore PPE properly when culling. Because the outbreak created an emergency situation and this study had not been designed before the outbreak, epidemiologic data were limited. And because we had insufficient serum for adsorption assays, we cannot exclude the possibility of cross-reactivity with circulating antibodies resulting from seasonal influenza vaccination or previous infection with human influenza virus. In 2004, among 83 Vietnam hospital employees who were exposed to 4 patients with confirmed and 1 patient with probable influenza (H5N1) virus infection, a positive antibody titer against influenza (H5N1) virus and cross-reacting antibodies against influenza (H1N1) virus was found on MN assay for 1 employee (*12*). Because our study was not a case–control study, we could not identify risk factors for transmission.

Regardless of these limitations, our study shows serologic evidence of influenza (H5N1) virus transmission among groups at high risk for poultry-to-human transmission (i.e., exposed to poultry during 2003–2004 outbreaks in South Korea). However, we also found additional proof that the frequency of poultry-to-human influenza (H5N1) virus transmission is low.

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# *Bartonella vinsonii* subsp. *arupensis* in Humans, Thailand

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We identified *Bartonella vinsonii* subsp. *arupensis* in pre-enriched blood of 4 patients from Thailand. Nucleotide sequences for transfer-messenger RNA gene, citrate synthase gene, and the 16S–23S rRNA internal transcribed spacer were identical or closely related to those for the strain that has been considered pathogenic since initially isolated from a human in Wyoming, USA.

More than 30 species of bartonellae that are highly prevalent in a wide variety of vertebrates have been described. *Bartonella bacilliformis*, *B. henselae*, and *B. quintana* are well-known human pathogens, and several other *Bartonella* species, including *B. elizabethae*, *B. tamiae*, *B. vinsonii* subsp. *arupensis*, have been associated with various clinical manifestations in humans (*1–3*). A link between some of these pathogenic strains and their animal hosts has been documented; for example, *B. elizabethae* is linked with *Rattus* rats. However, the reservoir host is unknown for other species, such as *B. tamiae*, which was isolated in patients from Thailand (*2*).

*B. vinsonii* subsp. *arupensis* was first isolated from a bacteremic cattle rancher in Wyoming, USA, in 1999 (*3*). Later studies showed that strains identical to *B. vinsonii* subsp. *arupensis* were highly prevalent among deer mice (*Peromyscus maniculatus*), a strictly North American rodent species frequently found across a wide geographic area, including Wyoming. Similar strains of *B. vinsonii* subsp. *arupensis* have not been found in other animals in North America, suggesting that deer mice are natural hosts of this bacterium (*4*).

However, the proposed link between infected mice and *B. vinsonii* subsp. *arupensis* infection in humans was challenged when this bacterium was reported in an endocarditis patient in France (*5*) and 2 febrile patients in Russia (*6*). The link was further disputed after identification of *B. vinsonii* subsp. *arupensis* infection in 2 humans in Thailand (*7*) and the subsequent inability to identify this strain or related species among the local rodent population, despite intensive investigation in different parts of Thailand (*8*). *B. vinsonii* subsp. *arupensis* was also identified in stray dogs in Thailand (*9*). In addition, *B. vinsonii* subsp. *arupensis*–specific antibodies were reported in febrile patients from Nepal (*10*). Together, these reports suggest that the spectrum of animal hosts carrying *B. vinsonii* subsp. *arupensis* may be underestimated. We report the identification of *B. vinsonii* subsp. *arupensis* in 4 more patients in Thailand.

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## The Study

The patients were enrolled in a febrile illness study in 4 rural hospitals in Chiang Rai and Khon Kaen Provinces, in northern and northeastern Thailand, respectively, during February 2002–March 2003. One of the patients (no. 45-00250) was enrolled as an afebrile patient. The 4 patients had some common clinical symptoms, such as headache, myalgias, dizziness, and fatigue. In addition, 3 of the patients had elevated levels of liver enzymes. All patients reported trapping or killing rats or seeing rats inside or around their houses during the 2 weeks before onset of symptoms, and all patients owned a dog and/or cat (Table).

To determine if the patients were infected with *Bartonella* species, we tested blood clots from each patient for the presence of the bacterium. For testing, we used a liquid growth medium (*Bartonella* α-*Proteobacteria* growth medium [BAPGM]) as a pre-enrichment step (*11*), improved molecular assays for *Bartonella* detection (*12*), and established molecular methods. The blood clots were inoculated into freshly prepared BAPGM. To avoid potential contamination, we did not supplement the medium with animal blood. Blank BAPGM controls were included for each inoculation. The inoculants and the blank controls were incubated aerobically at 35°C in 5% $CO_2$ for 7 days.

DNA was extracted from this pre-enriched medium by using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA). DNA was analyzed following published protocols (*12–14*). In brief, real-time PCR, nested PCR, and conventional PCR were used to target *Bartonella*-specific regions in the transfer-messenger RNA (*ssrA*) gene, the citrate synthase (*gltA*) gene, and the 16S–23S rRNA internal transcribed spacer, respectively. Positive controls (*B. doshiae*) and negative controls were included within each PCR run to evaluate the presence of appropriately sized amplicons and contamination, respectively. Amplicons were recovered from PCRs by gel purification (QIAGEN) and sequenced in both directions.

Table. Clinical characteristics and animal exposures of 4 patients with *Bartonella vinsonii* subsp. *arupensis* detected in blood, Thailand, February 2002–March 2003*

| Patient ID no. | Age, y/sex | Occupation | Province | Clinical symptoms | Abnormal laboratory test results | Pet(s) |
|---|---|---|---|---|---|---|
| 45–00025 | 56/M | Farmer | Chiang Rai | Fever, chill, fatigue, myalgia | AST 70 IU/dL, ALP 210 IU/dL | Cat |
| 45–01217 | 11/F | Student | Khon Kaen | Fever, chill, fatigue, myalgia, headache, icterus | Hb 11.3 g/dL, ALP 663 IU/dL | Cat, dog |
| 45–01239 | 39/F | Business | Khon Kaen | Fever, chill, fatigue, myalgia, headache, eye pain | Hb 12.1 g/dL, AST 73 IU/dL, ALT 141 IU/dL, ALP 388 IU/dL | Dog |
| 45–00250 | 35/M | Day laborer | Khon Kaen | Fatigue, myalgia, joint pain, headache, cough, abdominal pain | Normal values | Dog |

*All patients reported exposure to rats during the 2 weeks before onset of symptoms. ID, identification; AST, aspartate aminotransferase (reference value 5–35 IU/dL); ALP, alkaline phosphatase (reference value 30–115 IU/dL); Hb, hemoglobin (reference value <13.8 g/dL); ALT, alanine aminotransferase (reference value 5–35 IU/dL).

Sequences for the 3 targets were similar to those for the type strain of *B. vinsonii* subsp. *arupensis*. The *gltA* sequences (338 bp) from all 4 patient samples were identical to those for previously reported variants from a febrile patient in Thailand (GenBank accession no. GQ200857) and from stray dogs in Thailand (GenBank accession no. FJ946836). These *gltA* sequences were 0.8% divergent from the type strain of *B. vinsonii* subsp. *arupensis* (GenBank accession no. AF214557).

The *ssrA* sequences (251 bp) revealed 2 similar variants. One variant was identical to the type strain of *B. vinsonii* subsp. *arupensis* (GenBank accession no. JN029783) and was identified in samples from 3 of the patients (45-00250, 45-01217, and 45-01239). The other variant (GenBank accession no. JN394654), from patient 45-00025, was 2.8% divergent from the type strain of *B. vinsonii* subsp. *arupensis.*

In addition, two 16S–23S rRNA internal transcribed spacer variants were identified. One variant (GenBank accession no. JN402327), identified in samples from 3 patients (45-00025, 45-01217, and 45-01239), was 0.4% divergent from the type strain of *B. vinsonii* subsp. *arupensis* (GenBank accession no. AF312504). The other variant (GenBank accession no. JN402328), from patient 45-00250, was 0.9% divergent from the type strain of *B. vinsonii* subsp. *arupensis* (GenBank accession no. AF312504).

## Conclusions

In Thailand, at least 2 *Bartonella* species, *B. henselae* and *B. tamiae,* have been reported in association with human diseases (*2,15*). In addition, *Bartonella* DNA was detected recently in acutely ill patients from Thailand (*7*). *gltA* sequences revealed a broad range of *Bartonella* species in these patients, including *B. elizabethae*, *B. tribocorum*, *B. rattimassiliensis*, and *B. vinsonii* subsp. a*rupensis*. This finding suggests that numerous species of *Bartonella* may be associated with acute illness in Thailand.

In the current study, sequence data from 3 genetic targets provide additional evidence to confirm infection with *B. vinsonii* subsp. *arupensis* in patients in Thailand. Although the potential source(s) and mechanism(s) of transmission of this bacterium to humans remain unclear, the previous finding of *B. vinsonii* subsp. *arupensis* among stray dogs in Thailand (*9*) and the fact that 3 of the 4 patients in this report owned dogs could suggest dogs might be a source of the bacterium. Commonality of rat exposure may suggest that rats can also be potential reservoir hosts of *B. vinsonii* subsp. *arupensis*. Further investigation is needed in this regard.

It is known that diseases of humans and animals can translocate across the globe. For example, bubonic plague, caused by *Yersinia pestis*, was spread by infected rats on ships traveling from Asia to other continents. *Bartonella elizabethae* and other rat-associated *Bartonella species* that originated in the Old World have been similarly translocated to the New World and other regions (*1,8*). Reports of infections caused by *B. vinsonii* subsp. *arupensis* in humans in France, Russia, and Nepal, suggest that the role of this bacterium as a human pathogen may be more geographically widespread than previously believed.

Given the postulated New World origin of this pathogen, its ecology might serve as a model system to examine a possible translocation of *Bartonella* species across broad geographic regions and even between hosts. In addition, our finding of *B. vinsonii* subsp. *arupensis* in patients in Thailand suggests that this pathogen may be responsible for some undiagnosed cases of febrile illness and other types of illnesses in Thailand and possibly other countries in Asia.

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# Immunodeficiency-associated Vaccine-Derived Poliovirus Type 3 in Infant, South Africa, 2011

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Patients with primary immunodeficiency are prone to persistently excrete Sabin-like virus after administration of live-attenuated oral polio vaccine and have an increased risk for vaccine-derived paralytic polio. We report a case of type 3 immunodeficiency-associated vaccine-derived poliovirus in a child in South Africa who was born with X-linked immunodeficiency syndrome.

Live-attenuated oral polio vaccine (OPV) is still the vaccine of choice for use in developing countries. However, reversion to virulence may occur during OPV replication in humans and may result in the rare cases of vaccine-associated paralytic poliomyelitis in OPV recipients and their close contacts. Two additional OPV-related problems that may affect polio eradication: long-term, persistent infection with OPV-derived viruses in persons with primary humoral immunodeficiencies (so-called immunodeficiency-associated vaccine-derived polioviruses [iVDPVs]); and circulating vaccine-derived polioviruses (VDPV) in areas with low rates of vaccine coverage (*1*). VDPV strains are defined as follows: 1) strains of types 1 and 3, which have <99% nt sequence identity to the capsid viral protein (VP) 1 coding region of the corresponding Sabin reference strain; and 2) VDPV strains of type 2, which have <99.4% nt sequence identity to the corresponding Sabin reference viral protein 1 (VP1) (*1*). Circulating VDPVs show marked sequence drift, indicating prolonged replication of the vaccine strain in susceptible human hosts and consequent acquisition of the phenotypic properties of neurovirulence and transmissibility.

Persons born with primary immunodeficiency have been found to be persistently infected with VDPV after exposure to OPV. Immunocompetent persons excrete polio vaccine viruses for up to 2–3 months (*2*), whereas prolonged excretion of VDPV for 6 months to >10 years has been found in persons with primary humoral immunodeficiency (*3–6*). The risk for vaccine-associated paralytic poliomyelitis is >3,000-fold higher for these patients (*7*). We report a case of type 3 iVDPV in a child in South Africa who was born with X-linked immunodeficiency syndrome.

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## The Patient

The patient, a 10-month-old boy, was born at term on October 28, 2010; X-linked immunodeficiency syndrome was diagnosed after he received 3 scheduled doses of polio vaccine (1 OPV dose at birth and 2 inactivated poliovirus vaccine doses at 10 and 14 weeks). On September 18, 2011, fever developed (38.5°C–40.0°C), and the next day, vomiting and 2 episodes of tonic-clonic convulsions occurred. A lumbar puncture was performed, and testing of cerebrospinal fluid (CSF) showed pleocytosis and mild increase of proteins. His condition deteriorated, and on day 5, acute flaccid paralysis developed, with generalized hypotonia and reduced power and reflexes in all limbs, more marked in the lower limbs. Respiratory distress developed, and some involvement of the facial nerve was manifested by left-sided eye drooping, mouth deviation, and drooling. A lumbar puncture was repeated on day 5, and CSF was positive by PCR for enterovirus and a pleocytosis. Stool samples taken on days 5 and 9 were positive for enterovirus, which was subsequently characterized as poliovirus type 3.

Beginning 15 days after the onset of paralysis, intravenous immunoglobulin (National Bioproducts Institute, KwaZulu-Natal, South Africa) with a titer for polio type 3 neutralizing antibodies of 4–8 IU was administered daily for 32 days, followed by alternate days to a total of 43 doses. The patient improved gradually, and strength was regained in all limbs, with the exception of residual paresis in the right lower limb. CSF became negative for poliovirus PCR 2 weeks after immunoglobulin therapy began, and stool excretion of poliovirus ceased on day 70, 55 days after initiation of immunoglobulin therapy.

Extracts of stool specimens were treated with chloroform and cultured on human rhabdomyosarcoma cell line, used for enterovirus isolation, and mouse L cells expressing the human poliovirus receptor, used specifically for poliovirus isolation (*8*). To distinguish whether the poliovirus isolates were of vaccine or wild origin, real-time PCR tests were performed, targeting the VP1 coding region (*9*). In addition, to detect mutant and recombinant poliovirus vaccine strains, a vaccine-derived, real-time screening assay was performed (David Kilpatrick, pers. comm.).

All Sabin 3 strains were sequenced at 3 regions of the genome: 5′ untranslated region, VP1, and 3D. The sequence analysis of all viruses revealed a mutation at nt 472 of the 5′ untranslated region ($U_{472}$→C), a critical attenuating mutation feature for Sabin 3. This substitution

Table. Mixed bases at various positions of immunodeficiency-associated vaccine-derived polioviruses

| Sabin 3 | Viral protein 1 position | Mixed bases | |
|---|---|---|---|
| | | Stool sample 1 | Stool sample 2 |
| G | 175 | G/A | G/A |
| A | 231 | A/G | A/G |
| T | 261 | C/T | C/T |
| G | 295 | G/A | G/A |
| C | 420 | C/T | C |
| C | 426 | T/C | T/C |
| A | 444 | G/A | G/A |
| A | 658 | A/G | A/G |
| A | 659 | A/G | G/A |
| G | 661 | G/A | G/A |
| G | 666 | A/G | G/A |
| C | 684 | T/C | T/C |

in the internal ribosomal site restores the original structure of the stem loop and permitting the initiation of translation of the poliovirus RNA template (*10*,*11*) The reversion at that site is under strong selection during replication in the human intestine and is associated with the attenuated phenotype in Sabin 3 (*12*). The VP1 region showed 2 reversions of the capsid determinant; $C_{2493}$→U appear to be the main determinants of the attenuated phenotype (*1*), and at position 54 for alanine amino acid mutated to valine ($Ala_{54}$→Val) that can act as a suppressor of the temperature sensitivity and attenuated phenotype (*13*). At the 3D region, the sequence analysis showed no recombinant.

Both stool samples showed mixed bases at 12 positions, consistent with the presence of at least 2 main genetic variants in the virus population (Table). Isolates with mixed bases are characteristic of iVDPVs, which suggests the existence of co-replicating poliovirus lineages within immunodeficient patients (*1*,*5*).

The relationships among the VP1 sequences of the 3 isolates were summarized in a tree constructed by using the neighbor-joining method (*14*) and rooted to the Sabin 3 sequence (Figure). The iVDVP isolates differed from the Sabin 3 OPV strain at 1.1% and from each other by 1.4% at a VP1 region, similar to the rate of nucleotide sequence evolution in poliovirus as described by Jorba et al. (*15*). The chronic iVDPV infection could have been initiated by the birth dose. The shallow branches correspond to 2 lineages (A, CSF, and B, stool). The extensive divergence of the two lineages was not surprising as the viruses originated from 2 sources (CSF and stool samples) taken 4 days apart. The VP1 sequence of lineage B was ambiguous at several positions, which suggests the virus population was of mixed variants. All sequences determined in this study were derived from Sabin 3 strain.

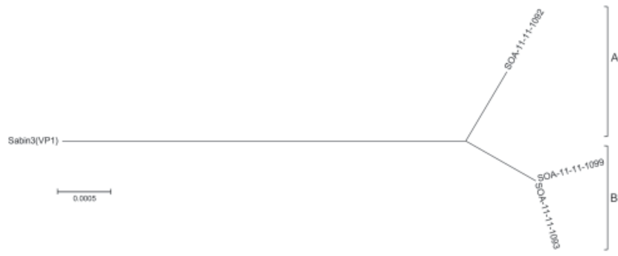


Figure. Neighbor-joining tree of immunodeficiency-associated vaccine-derived poliovirus isolates from infant, South Africa, 2011. The tree was derived from the viral protein (VP) 1 region and rooted at the Sabin 3 reference strain, showing classification as lineage A or B. South Africa (SOA) isolates are identified by 2 digits indicating year of receipt, 2 digits indicating year of onset of paralysis (*11*), and 4 digits indicating case number. SOA-11-11-1092 is a representative cerebrospinal fluid sample; SOA-11-11-1099 and SOA-11-11-1093 are representative stool samples. Scale bar indicates nucleotide substitutions per site.

## Conclusions

Cases of iVDPV are rare; especially rare is type 3. Only ≈50 cases had been reported in the literature as of March 2011 and, to our knowledge, none in sub-Saharan Africa. We characterized 2 separate lineages of type 3 poliovirus in this patient, demonstrating separate evolution of the virus. A relatively rapid clinical and virologic response to intravenous immunoglobulin averted chronic excretion of the virus. Persistent excretion of VDPV in primary immunodeficient patients remains a potential risk to the global eradication of polio, as long as OPV is still used.

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# *Rickettsia parkeri* Infection in Domestic Dogs, Southern Louisiana, USA, 2011

**Britton J. Grasperge, Wendy Wolfson, and Kevin R. Macaluso**

The association between companion animals and tick-borne rickettsial disease has long been recognized and can be essential to the emergence of rickettsioses. We tested whole blood from dogs in temporary shelters by using PCR for rickettsial infections. Of 93 dogs, 12 (13%) were positive for *Rickettsia parkeri*, an emerging tick-borne rickettsiosis.

Tick-borne spotted fever group (SFG) rickettsioses are maintained in tick populations through vertical transmission of the rickettsial agent and horizontal transmission among vectors by a vertebrate host. Companion animals, specifically dogs, can serve as vertebrate hosts for arthropod vectors and SFG rickettsia (*1*), as shown by a report of a *Rickettsia parkeri*–infected dog in South America (*2*). Likewise, cases of rickettsioses in humans have been associated with cases in companion animals (*3*). Because of a substantial increase in tick-borne rickettsial diseases in the past decade, much effort has been directed to identifying the rickettsial agents present in ticks (*4*). On the basis of findings from field surveys of rickettsial infections in ticks and characterization of rickettsioses in humans, most cases of what is considered Rocky Mountain spotted fever, a disease caused by *R. rickettsii*, are likely caused by infections with rickettsial species other than *R. rickettsii* (*5*).

One of the better documented emerging rickettsial pathogens is *R. parkeri*, an SFG tick-borne rickettsial disease associated with Gulf Coast ticks (*Amblyomma maculatum*) (*6*) and commonly identified in the coastal states of the southeastern United States. We investigated the potential role domestic dogs play in the ecology of *R. parkeri* transmission to better understand the epidemiologic landscape of this emerging rickettsiosis.

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## The Study

We obtained blood from dogs at 5 animal control centers in 5 parishes in southern Louisiana during June and July 2011. The blood for the study was provided from excess samples collected for routine heartworm screening. In total, 93 dogs were included in the study. Within 12 hours of collection, whole blood samples (≈50–100 μL) were processed individually for DNA extraction by using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA). DNA was stored at –20°C until PCR analysis.

DNA extracts from the collected blood, environmental DNA extraction controls, or water (negative controls) were used as template for PCR. PCR products were amplified by using genus-specific 17-kDa antigen gene primers and described thermocycling conditions (*7*). Amplicons were visualized by electrophoresis on 2% agarose gels. Positive samples were excised from the gels, and the amplicons were purified by using the PCR Clean-Up System (Promega, Madison, WI, USA). Positive samples were sequenced, and sequences were aligned by using MEGA5.05 (http://megasoftware.net/mega.php), and nucleotide similarities were assessed by using the GenBank BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

DNA samples positive for *Rickettsia* spp. by the genus-specific 17-kDa antigen gene primers were also assessed for the SFG-common rickettsial outer membrane protein A gene (*rompA*) by using a heminested PCR with primers 190.70p and 190.701 followed by primers 190.70p and 190.602n. Primers and thermocycling conditions for the heminested PCR were as described (*7*), and subsequent purification and sequencing were performed as described above.

Of the 93 DNA samples, 12 (≈13%) produced positive amplicons for the genus-specific 17-kDa antigen gene. On the basis of sequence data, the positive samples were determined to be most closely related to SFG rickettsiae. The resulting 315-bp sequence showed 100% identity to *R. montanensis* (GenBank accession no. DQ402377.1) and 99% identity to several other members of the SFG including *R. rickettsii*, *R. parkeri*, *Candidatus* Rickettsia andeanae, and *R. sibirica* (GenBank accession nos. CP000766.2, EF689732.1, GU395295.1, and AF445384.1, respectively).

The heminested PCR for *rompA* yielded a 491-bp product with identical sequences for each of the 12 *Rickettsia*-positive samples. Sequence analysis of the *rompA* amplicon identified a 99% similarity with several different strains of *R. parkeri* (GenBank accession nos. U43802.1, EU715288.1, EF102238.1, FJ172358.1, and HM587252.1). These *Rickettsia*-positive samples were obtained from 3 of the 5 sites surveyed, and 2 of the 3 sites were in parishes that directly adjoined each other (Table). Within the dog populations tested in the 3 sites, 22% (2/9), 16% (9/55), and 8% (1/12), respectively, of the dogs were infected with *R. parkeri* (Table).

Table. *Rickettsia parkeri* infection in domestic dogs in 5 animal shelters from 5 parishes in southern Louisiana, USA, June and July 2011*

| Parish | No. dogs positive/no. tested (%) |
|---|---|
| Ascension | 0/13 |
| Livingston† | 2/9 (22) |
| Iberville | 0/4 |
| East Feliciana | 9/55 (16) |
| Tangipahoa† | 1/12 (8) |

*The presence of *R. parkeri* was determined by PCR for *rompA*. *R. parkeri*, *Rickettsia parkeri*; rompA, rickettsial outer membrane protein A.
†Parishes directly adjoin one another.

None of the 12 dogs with PCR-positive tests were infested with ticks at the time of sampling. Six female dogs and 6 male dogs had detectable levels of *R. parkeri* DNA in their blood. Nine of the 12 dogs were adults; 3 were <6 months of age. Many of the dogs in the study were classified as mixed breed because breed could not be objectively determined for most of the animals. All animals appeared to be in good health; no overt pathology was noted at the time of blood collection.

Although molecular detection of rickettsial DNA within the blood of vertebrates indicates infection, rickettsial cultures from the positive samples would confirm patent rickettsemia. Most of the samples in our study were insufficient in volume to attempt culture after heartworm testing and DNA extraction. Of the 12 samples with PCR results positive for rickettsial DNA, only 3 were of sufficient volume to attempt culture, and all of those attempts proved unsuccessful. It would also have been beneficial to determine if dogs that were positive for *R. parkeri* harbored ticks that were also positive for *R. parkeri*. However, it is common practice for animal control centers to treat dogs for ectoparasites on admission; thus, no ticks were present on the dogs in our study at the time of sampling. The presence of rickettsial DNA in the blood of dogs, in the absence of ectoparasites, supports the hypothesis that domestic canines may serve as reservoirs of rickettsial diseases, now specifically including the emerging pathogen *R. parkeri*.

## Conclusions

We examined the potential role of domestic dogs in transmission of SFG *Rickettsia*. *R. rickettsii*, the causative agent of Rocky Mountain spotted fever, is known to cause clinical disease in dogs, and it is associated with signs and symptoms that are similar to human disease, including cutaneous petechiae and ecchymoses, anorexia, depression, weight loss, and dehydration (*8*). The role of dogs as vehicles for *Rickettsia*-infected ticks to encounter susceptible humans has also been proposed (*1*). The possibility of dogs as reservoirs of rickettsial disease has previously been investigated in studies evaluating *R. felis* rickettsemia and seropositivity for *R. parkeri* (*9,10*); however, strong cross-reaction among antibodies precludes finding of definitive results from serologic testing. The current study suggests that domestic dogs may become rickettsemic with *R. parkeri* infection, but further investigation of the duration of rickettsemia and monitoring for clinical disease that may be associated with infection is required.

It is also vital to determine the potential for dogs to serve as infectious sources of *R. parkeri* for feeding ticks. Dogs infected with *R. rickettsii*, for example, have proven relatively inefficient at transmitting rickettsiae to naive ticks and therefore may not play a large role in maintenance or amplification of the *R. rickettsii* transmission cycle (*11*). Conversely, domestic dogs have recently been shown to be competent reservoirs for the causative agent of Mediterranean spotted fever, *R. conorii*, a species closely related to *R. parkeri* (*12*). The prevalence identified in this study establishes an important first step in the examination of the domestic dog for reservoir competency of *R. parkeri*.

Since the first reported case of *R. parkeri* rickettsiosis in 2004, >20 additional cases have been identified in humans (*13*), and to date no viable vertebrate reservoirs for the pathogen have been identified. Although the current study consists of a relatively small survey, the results are considerable because of the recognized importance of domestic dogs as potential reservoirs for transmissible pathogens (*14*). In addition, the presence of *R. parkeri* has not previously been described in Louisiana; thus, this report expands the known distribution of *R. parkeri*. The results of the current study clearly establish dog infection by *R. parkeri*; however, a role for dogs in the natural cycle of this pathogen, and the arthropod vectors involved in transmission, requires further investigation.

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Dr Grasperge is a veterinary clinical pathologist and predoctoral candidate in the Vector Biology Research Laboratory at Louisiana State University School of Veterinary Medicine. His work focuses on the pathogenesis of eschar-associated rickettsioses.

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# etymologia

## Syphilis

**[ˈsi-f(ə-)ləs]**

From *Syphilis sive morbus gallicus* ("Syphilis or the French disease") (1530) by Italian physician and poet Girolamo Fracastoro. The poem tells of Syphilus, a shepherd who insulted the sun god of Haiti. In retaliation, the god sends a plague to Haiti, and Syphilus is the first victim.

The first recorded syphilis epidemic was in 1495, during the First Italian War. After the French captured Naples, disbanded soldiers spread syphilis across Europe. For nearly 500 years, scholars have argued whether Columbus brought syphilis to Europe from the New World. Recent research supports Fracastoro's New World origin for the disease.

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# Wild Boars as Hosts of Human-Pathogenic *Anaplasma phagocytophilum* Variants

**Jerzy Michalik, Joanna Stańczak, Stella Cieniuch, Maria Racewicz, Bożena Sikora, and Mirosława Dabert**

To investigate the potential of wild boars to host *Anaplasma phagocytophilum,* we analyzed bacterial 16S rRNA and *ank* genes. DNA sequencing identified several *A. phagocytophilum* variants, including a predominance of strains known to cause human disease. Boars are thus hosts for *A. phagocytophilum*, notably, strains associated with human granulocytic anaplasmosis.

The enzootic cycle(s) of *Anaplasma phagocytophilum*, a tick-transmitted bacterium that causes granulocytic anaplasmosis (GA) in humans (HGA) and certain domesticated animals is driven by the distribution of its vector ticks and wild mammal reservoirs (*1*). Molecular and phylogenetic analyses of *A. phagocytophilum* sequences from ticks and hosts provide evidence that this bacterium comprises a complex of closely related strains that differ in their host preferences and pathogenicity (*2–4*). Although 16S rRNA, *groESL*, and *ank* gene variants from horses with GA in Europe, and less frequently from infected dogs, are identical to sequences from most HGA patients (*4–7*), the wild reservoir hosts for strains causing human anaplasmosis (AP-ha) in Europe are poorly understood.

In contrast to the eastern United States, where white-footed mice are a primary reservoir for strains that infect humans, rodents in Europe have not been found to display high zoonotic potential (*8*). Moreover, cervids have been found to propagate mostly *A. phagocytophilum* variants that have not been detected in humans (*9*,*10*). An exception to this finding is that red deer seem to maintain strains that induce HGA (*4*). In Slovenia, identical *A. phagocytophilum groESL* sequences have been identified in patients and wild boars (*Sus scrofa*), which suggests that boars may represent a potential reservoir for AP-ha variants (*10*,*11*). Although several clinical cases of HGA have been reported in Poland (*12*), no data are available concerning *A. phagocytophilum* infections in boars, even though they are the most abundant big game animals (≈200,000 animals are hunted and killed annually) and host all 3 parasitic stages of the tick vector *Ixodes ricinus*. Thus, we sought to determine the frequency of *A. phagocytophilum* in populations of wild boars and in host-derived ticks to clarify the role of boars in the ecology and epidemiology of GA.

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## The Study

Sampling was performed at 2 tagging stations, Zielonka and Kąty, in distinct forested areas situated within the Zielonka Primeval Forest, in west-central Poland. EDTA-blood specimens were collected from 325 animals harvested during May–December 2006, 2007, and 2008. The animals represented 3 age groups: piglets (34%), yearlings (49%; >1 to 2 years), and adults (17%). Paired samples of liver and blood were collected from 24 boars. During May–November 2006, 50 animals were inspected for ticks at Zielonka. DNA was extracted from blood and ticks by using Genomic Mini AX Blood and Sherlock AX kits (A&A Biotechnology, Gdynia, Poland). Nested PCR targeting a 546-bp fragment of the *A. phagocytophilum* 16S rRNA gene was performed (*13*). Selected positive samples were subjected to a second PCR targeting a 444-bp region of the *ankA* gene (*14*).

Selected 16S rRNA and *ankA* PCR amplicons were sequenced with an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and analyzed by using BLASTn (www.ncbi.nlm.nih.gov.blast) analysis of GenBank sequences. Phylogenetic dendrograms were constructed by the neighbor-joining algorithm method (Vector NTI Advance version 10.3.0, Invitrogen Corp., Carlsbad, CA, USA). Nine partial 16S rRNA and 6 *ankA* sequences detected were deposited in GenBank under accession nos. GU391312–GU391320 (7 from boars, 2 from ticks) and GU434664–GU434669 (4 from boars, 2 from ticks), respectively.

Of the 325 animals tested, 39 (12%) yielded *A. phagocytophilum* 16S rRNA amplification products (Table). Bacteremic hosts were detected in all 3 years with the highest prevalence (20.3%) recorded in 2006 and the lowest (8.8%; $\chi^2$ test, p = 0.015) in 2008. Bacterial DNA was identified in all 3 host-age groups, i.e., in 13.6% of piglets, 12.6% of yearlings, and in 7.1% of adults. The overall infection prevalence among animals from Zielonka (17.4%) was significantly higher than among those from Kąty (7.1%; p = 0.004). The finding that 26% of the 50 animals harvested at Zielonka in 2006 were infected represents the highest *A. phagocytophilum* infection prevalence ever recorded among boars. Of 24 liver samples, 2 (8.3%) tested positive. Both animals with positive liver

Table. *Anaplasma phagocytophilum* infection in wild boars surveyed at 2 tagging stations in west-central Poland from mid-May to December, 2006–2008

| | No. positive/no. tested (%) | |
|---|---|---|
| Year | Zielonka | Kąty |
| 2006 | 13/50 (26.0) | 3/29 (10.3) |
| 2007 | 7/48 (14.6) | 3/51 (5.9) |
| 2008 | 7/57 (12.3) | 6/90 (6.7) |
| 2006–2008 | 27/155 (17.4) | 12/170 (7.1) |

samples also yielded the bacterium in blood. The remaining 22 blood samples were PCR negative.

Seventy partially engorged *I. ricinus* ticks (58 nymphs, 11 females, 1 larva) were collected from 9 animals (7.8 ticks per infested animal). Because all of these ticks parasitized boars with negative blood specimens, the pathogen identified in 3 (5.2%) nymphs and 4 (36.4%) female ticks could have been acquired during their previous blood meal. On the other hand, detecting the same 16S rRNA variant in a female tick (294–9) and in a nymph (294–6; Figure 1) that fed side by side on a yearling, may indicate that the bacterium was acquired by co-feeding transmission (between infected and noninfected ticks).

Sequencing of 16S rRNA products from 27 selected animals (20 from Zielonka, 7 from Kąty) produced 29 sequences (27 from blood, 2 from liver). These sequences showed marked diversity, representing 7 different *A. phagocytophilum* variants that were 99.6% similar to each other. The most common variant comprised 13 (44.8%) sequences (e.g., 470–5, Figure 1) that matched sequences reported from HGA patients in North America and Europe, including a sequence from a patient in Poland (Figure 1). These sequences, which were related to sequences found in HGA case-patients, prevailed among infected animals (12 of 20) from Zielonka, which harbored them in all 3 years. The second most frequently amplified variant comprised 10 (34.5%) sequences identical to a sequence from red deer in Slovenia. Two hosts from Kąty yielded an AP-variant 1 strain for which white-tailed deer are reservoirs in the United States (*15*). Three distinct sequences matched sequences from a dog, a tick, and a mouflon in Germany, whereas 1 sequence (468–3) was unique. Two boars with positive liver samples (457; 717, Figure 1) had dual infections caused by distinct variants identified in blood and liver samples, respectively.

Among the 4 sequences from ticks, 2 clustered with the AP-ha variant. Twelve partial *ankA* sequences detected in 10 boars and 2 ticks showed 98.9% homology to each other. Seven (70%) animals yielded sequences (e.g., 470–5; Figure 2) identical to a sequence from an HGA patient in Sweden. Their corresponding 16S rRNA gene sequences clustered with the AP-ha variant. Of the remaining *ankA* sequences, 1 matched a sequence from a roe deer in Poland, whereas 2 were unique. These 3 sequences were identical on the basis of the 16S rRNA gene. This finding confirms that the *ankA* gene is a more informative marker for the characterization of genetic diversity in *A. phagocytophylum* (*4*). Among 2 *ankA* sequences obtained from ticks, a female tick yielded the predominant *ankA* variant, whereas a nymph harbored a novel variant.

## Conclusions

The presence of bacteremic animals (range 9%–20%) throughout this study provides compelling evidence for the involvement of wild boars in the enzootic cycle of *A. phagocytophilum*. Further studies with larger tick samples are necessary to investigate the efficiency of boar-to-tick transmission. The fact that most of the partial 16S rRNA

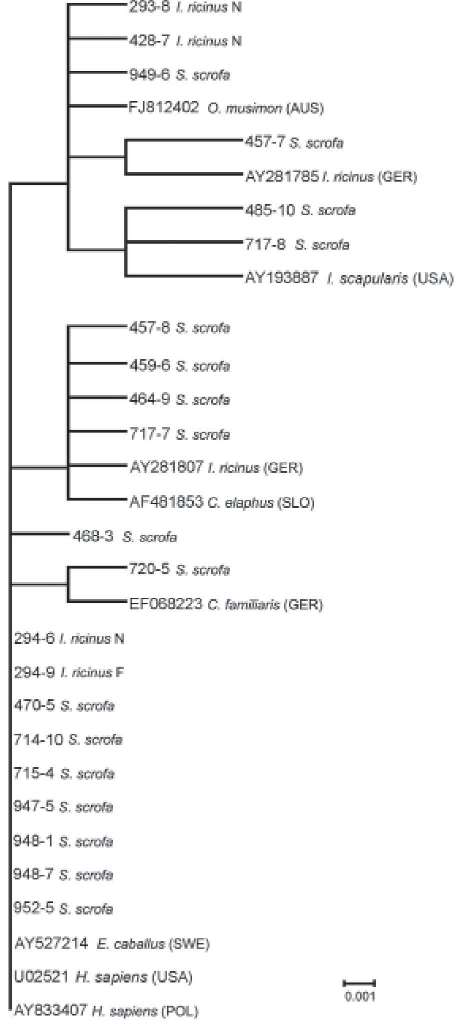


Figure 1. Phylogenetic relationships based on *Anaplasma phagocytophilum* 16S rRNA gene fragment sequences obtained from wild boars and engorged *Ixodes ricinus* ticks and selected sequences from GenBank. The scale bar indicates an evolutionary distance of 0.001 nt per position in the sequence. Inference was made by using the neighbor-joining algorithm method (Vector NTI Advance 10.3.0; Invitrogen Corp., Carlsbad, CA, USA).

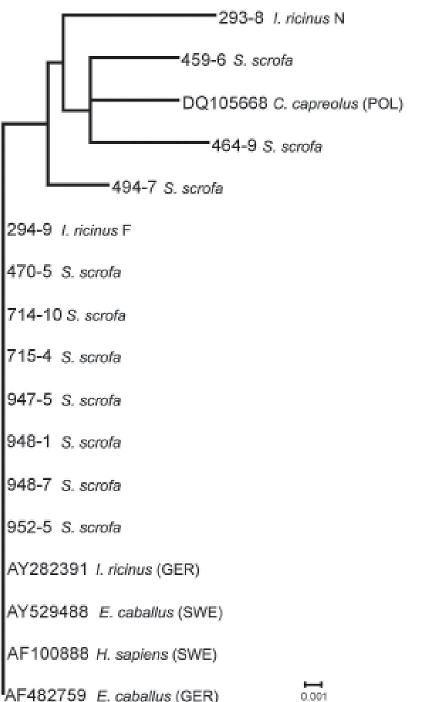


Figure 2. Phylogenetic relationships based on *Anaplasma phagocytophilum ankA* gene fragment sequences obtained from wild boars and engorged *Ixodes ricinus* ticks and selected sequences from GenBank. The scale bar indicates an evolutionary distance of 0.001 nt per position in the sequence. Inference was made by using the neighbor-joining algorithm method (Vector NTI Advance 10.3.010.3.0; Invitrogen Corp., Carlsbad, CA, USA).

and *ankA* sequences (13 of 27 and 7 of 10, respectively) amplified from boars corresponded to *A. phagocytophilum* strains known to cause human disease, reconfirms earlier findings in which *groESL* sequences identical to those from patients in Slovenia were found in wild boar (*S. scrofa*) populations in the Czech Republic and Slovenia (*10*,*11*). Detection of *A. phagocytophilum* strains associated with human infections in all 3 boar age groups in Zielonka, as well as in host-derived ticks, strongly implicates the wild boar as a notable host of HGA variants.

Because bacteremia among hosts from Zielonka was frequent (range 12%–26%) and boars are quite abundant in Europe, they could be used as sentinel animals for detecting *A. phagocytophilum* infections, notably strains known to be infectious for humans. Additional molecular and serologic studies including essential reservoir-competence experiments with AP-ha strains are needed to further elucidate the role of wild boars in the epidemiology of *A. phagocytophilum*.

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Dr Michalik is a researcher in the Department of Animal Morphology at Adam Mickiewicz University in Poznań. His research focuses on host-vector-pathogen relationships, with a particular emphasis on the ecology of tickborne diseases.

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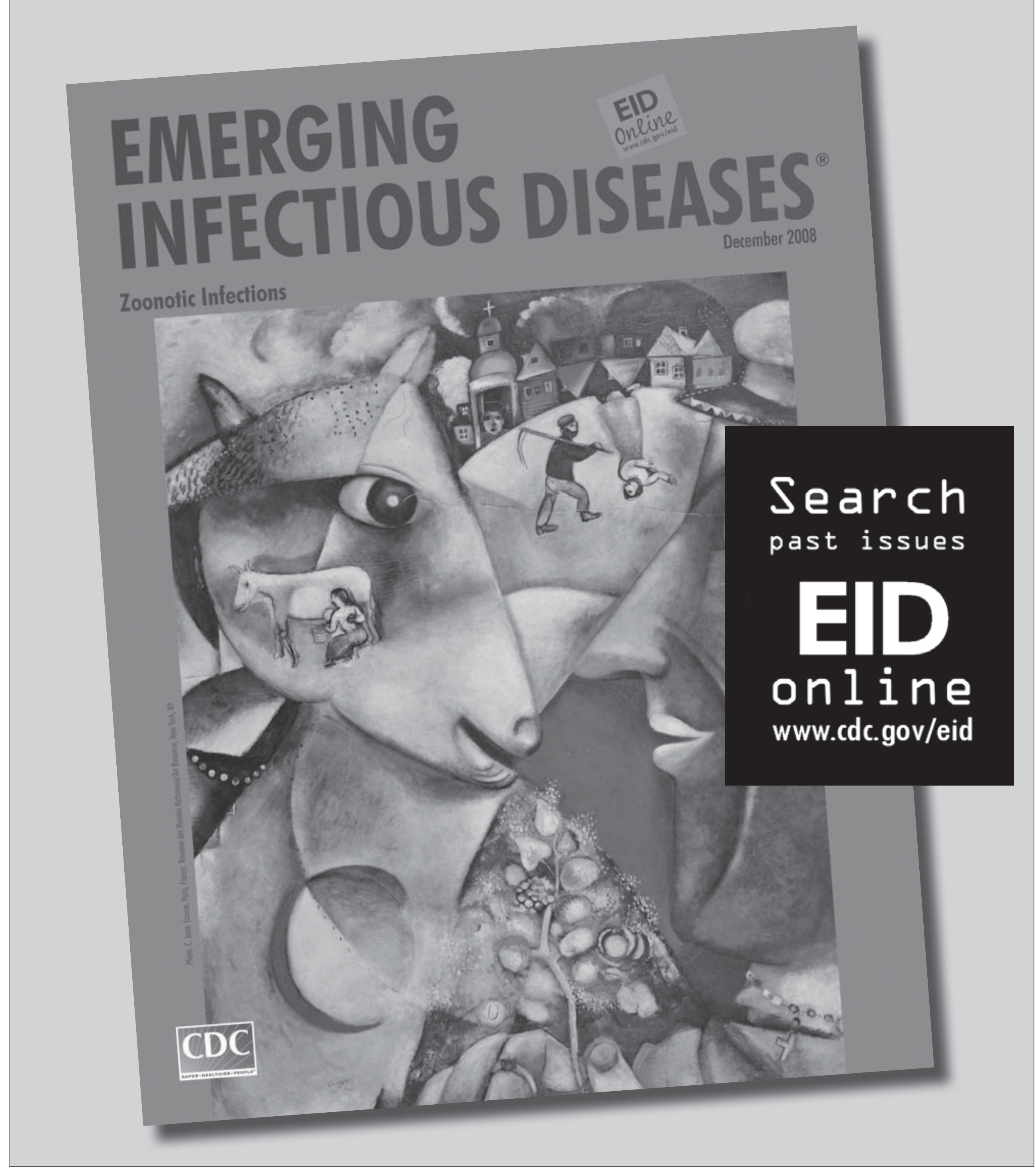

# Local Transmission of Imported Endemic Syphilis, Canada, 2011

**Sergio Fanella, Kamran Kadkhoda, Michelle Shuel, and Raymond Tsang**

Endemic (nonvenereal) syphilis is relatively common in nonindustrialized regions of the world. We describe a case of local transmission in Canada and review tools available for confirming a diagnosis. Improved molecular tools and global clinical awareness are needed to recognize cases of endemic syphilis imported to areas where it is not normally seen.

*Treponema pallidum* subsp. *endemicum* is the causative agent of endemic syphilis, also called nonvenereal syphilis. Other diseases caused by nonvenereal treponematoses are yaws (*T. pallidum* subsp. *pertenue*) and pinta (*T. carateum*). The 3 diseases are a substantial cause of illness in the nonindustrialized world, but they are rarely encountered in industrialized areas. Endemic syphilis is encountered in dry, hot regions, including Sahelian areas of western Africa and parts of Botswana, Zimbabwe, and the Arabian Peninsula (*1–4*). The causative organism is transmitted by direct contact with secretions from lesions or on fomites. The clinical spectrum of these diseases involves various degrees of involvement of the skin, mucous membranes, and skeletal system, depending on the organism (*1*,*2*,*5*,*6*).

## The Study

In February 2011, a 1-year-old girl was referred to our infectious diseases clinic for assessment of skin lesions. The patient had a history of several flesh-colored papules on her forehead at 2 months of age and on other areas of her body (hands, wrists, axilla, and anus) over the next several months. One week before our assessment, she began a 5-day course of azithromycin. Improvement in the lesions and resolution of an oral ulcer were reported by the parents at the assessment. The child appeared well. On examination in our clinic, flesh-colored papules were found on her right hand, wrist, and right axilla. A 1-cm condylomatous perianal lesion was also seen. The remainder of her examination findings were normal. Serologic screening for antibodies to *T. pallidum* was performed by using the Venereal Disease Research Laboratory (VDRL) test, which resulted in a 1:32 dilution, and the *T. pallidum* particle agglutination (TP-PA) assay of 4+ reactivity. Long bone radiographic results were normal.

The patient was born in Winnipeg, Canada, to a 39-year-old woman after a non-eventful pregnancy. The mother's antenatal serologic results were negative for HIV and hepatitis B, and for syphilis by negative VDRL and TP-PA test results. The family lived in a refugee camp in the Republic of Senegal for 20 years before immigrating to Canada in November 2009. Review of laboratory records and discussions with public health services revealed that most of the family members had VDRL and TP-PA tests at a community clinic in early 2010 for unknown reasons, but they were lost to follow-up. The clinic was permanently closed and records were not available. The family had not traveled nor received visitors from overseas since moving to Canada.

We assessed all family members. Results of their examination and testing are described in Table 1. The patient's 3-year-old brother had a 2-month history of progressive drooling and a hoarse voice. On examination, a 2 cm–diameter ulcer with raised edges and friable appearance was noted on the inside of his lower lip. He had a hoarse cry when agitated and drooled excessively, and he had palpable bilateral cervical lymphadenopathy. The remainder of his physical examination showed no abnormalities. His VDRL test result was positive at a 1:32 serum dilution, as was TP-PA with 4+ reactivity. PCR was performed on swab specimens from oral lesions by using previously described protocols (*7*) to detect the presence of 3 *T. pallidum* genes: *bmp*, *polA*, and *tpp47*. All 3 genes were detected.

To determine whether the 3-year-old boy was infected with venereal syphilis or for nonvenereal *T. pallidum*, PCR was performed on a sample. The acidic repeat protein (*arp*) gene was amplified from the clinical specimen by using PCR primers and conditions as described (*8*). DNA sequencing of the purified PCR amplicons showed that the *arp* gene contained a central region of eight 60-bp repeats; all repeats contained identical nucleotide sequences (GenBank accession no. JN674561). The translated amino acid sequence, REVEDVPKVVEPASEREGGE, is characterized as a Type II repeat motif and has been described only in *arp* genes from nonvenereal *T. pallidum* subspecies (*8*,*9*).

Additional differentiation was accomplished by identification of subspecies-specific signature sequences in the *tprI* and *tprC* genes (*10*): the genes were amplified from the clinical specimen and from syphilis control

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Table 1. Serologic testing results for *Treponema pallidum* subspecies *endemicum* and clinical findings for a symptomatic patient and family members, Canada, 2011*

| Family member† | Age, y/sex | VDRL result | TPPA result | Clinical findings | Molecular detection/PCR |
|---|---|---|---|---|---|
| Parent | 40/F | Negative | Negative | None | ND |
| Parent | 49/M | Negative | ND | None | ND |
| Child | 1/F | 1:32 | 4+‡ | Skin papules, perianal condylomata | –§ |
| Child | 3/M | 1:32 | 4+ | Oral ulcer, hoarse voice, drooling, adenopathy | +¶ |
| Child | 5/F | Weakly reactive | 4+ | None | ND |
| Child | 7/F | 1:2 | 4+ | None | ND |
| Child | 10/F | 1:8 | 4+ | None | ND |
| Child | 11/M | 1:4 | 4+ | None | ND |
| Child | 14/M | 1:16 | 4+ | None | ND |
| Child | 16/F | Weakly reactive | 4+ | None | ND |
| Child | 19/F | 1:16 | 4+ | None | ND |

*VDRL, Venereal Disease Research Laboratory test; TPPA, *T. pallidum* particle agglutination assay; ND, not done.
†All family members received intramuscular benzathine penicillin (1.2 million U if ≥10 y old, 0.6 million U if <10 y old) after confirmatory PCR results for the 3-year-old child.
‡Positive reactions were measured on a scale of 1–4, with 4 as the highest value.
§Oral swab specimen. Child had history of oral ulcer but had received a full course of azithromycin followed by oral lesion resolution before PCR was performed. She was born in Canada, had no travel history, and although the PCR result was negative, the test was performed after the patient received treatment. The patient had positive serologic test results for *T. pallidum*, a history of lesions characteristic of endemic syphilis, and no other plausible explanation for findings.
¶Oral ulcer swab specimen.

DNA prepared from *T. pallidum* subsp. *pallidum* Nichols strain by using PCR. Because DNA for *T. pallidum* subsp. *endemicum* and *T. pallidum* subsp. *pertenue* was not commercially available, it was not included in this study. The subspecies-specific signatures were identified by restriction fragment length polymorphism (*10*) and DNA sequencing.

To characterize the *tprI* gene, PCR amplicons were digested with the restriction enzyme *Bsr*DI (New England Biolabs, Pickering, Ontario, Canada). The *tprI* gene from the clinical specimen was digested into 2 fragments of 334 bp and 159 bp (Figure), whereas *tprI* amplicons from the syphilis control strain were not digested and remained as a single band of 493 bp. DNA sequencing of the *tprI* gene from the clinical specimen confirmed the presence of a *Bsr*DI site (NN/CATTGC at position 1759–1766), which is typical for *T. pallidum* subsp. *endemicum*; no restriction site was observed in the syphilis control strain or in GenBank sequences for *T. pallidum* subsp. *pertenue* (Table 2).

To characterize the *tprC* genes, we simultaneously digested PCR amplicons by using restriction enzymes *Bsr*DI and *Bsi*EI (New England Biolabs). Digestion of DNA from *T. pallidum* subsp. *endemicum* strains with *Bsr*DI yielded 2 bands, 547 bp and 160 bp. The syphilis control strain also showed bands at 547 bp and 160 bp after digestion with *Bsr*DI, because of a site found in only one *T. pallidum* subsp. *pallidum* strain (Figure). The restriction fragment length polymorphism patterns for the *tprC* genes from the clinical specimen and the syphilis control strain were identical (Figure). However, DNA sequencing of the *tprC* gene from the clinical specimen revealed a >99% match to *tprC* GenBank sequences from other *T. pallidum* subsp. *endemicum* strains, a ≈97% match to *T. pallidum* subsp. *pallidum* Nichols strain (the syphilis control strain), and a ≈98% match to GenBank sequences for *T. pallidum* subsp. *pertenue* (Table 2). Furthermore, DNA sequence confirmed the presence of a *Bsr*DI restriction site in the *tprC* gene, which would be absent in subspecies *pertenue*. Characterization of the *arp*, *tprI,* and *tprC* genes identified

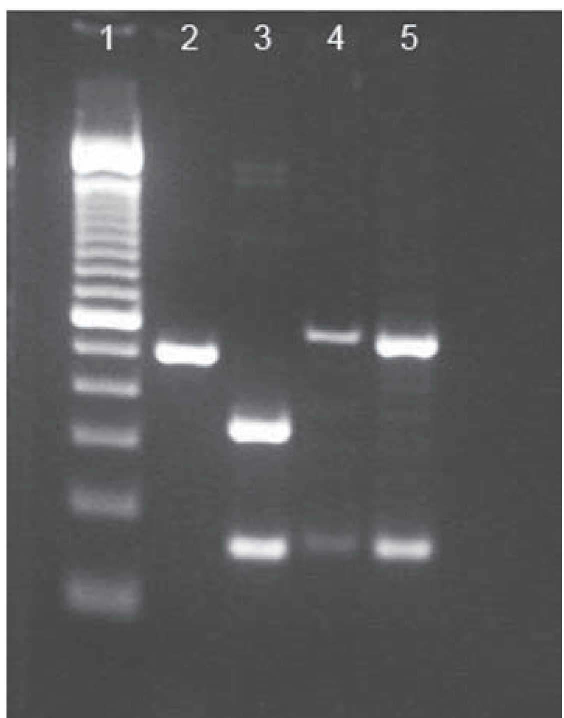


Figure. *Bsr*DI digest of *tprI* and *Bsr*DI/*Bs*EI double digest of *tprC* from the oral ulcer swab specimen and the syphilis control strain Nichols. Lanes from left to right: 1, 100-bp ladder; 2, Nichols *tprI* product not digested with BsrDI (493-bp band); 3, clinical specimen *tprI* product digested with *Bsr*DI (334-bp and 159-bp bands); 4, Nichols *tprC* digested with *Bsr*DI/*Bsi*EI (547-bp and 160-bp bands); 5, clinical specimen *tprC* digested with *Bsr*DI/*Bsi*EI (547-bp and 160-bp bands).

Table 2. Comparison of nucleotide sequences of *tprI* and *tprC* genes detected in clinical specimens from cases of endemic syphilis, Canada, 2011*

| Strain† | *tprI* gene sequence at nt positions 1740–1766, 5′ → 3′ | GenBank accession no. |
|---|---|---|
| Clinical strain | **CT**C **C**GA **TGT** TCC **C**TA CAT GGG **C**AT TGC | JN674562 |
| Bosnia A | **CT**C **C**GA **TGT** TCC **C**TA CAT GGG **C**AT TGC | DQ886678 |
| Nichols‡ | **TG**C **T**GA **CGC** TCC **T**TA CAT GGG **T**AT TGC | NC_000919 |
| Samoa D | **TG**C **T**GA **CGC** TCC **T**TA CAT GGG **T**AT TGC | DQ886680 |
| | *tprC* gene sequence at nt positions 1704–1736, 5′ → 3′ | |
| Clinical strain | **G**GT GCT **C**TC **C**GA **TGT** TCC **C**TA CAT GGG **C**AT T**G**C | JN674563 |
| Bosnia A | **G**GT GCT **C**TC **C**GA **TGT** TCC **C**TA CAT GGG **C**AT T**G**C | DQ886673 |
| Nichols‡ | **C**GT GCT **T**GC **T**GA **CGC** TCC **T**TA CAT GGG **C**AT T**G**C | NC_000919 |
| Samoa D | **G**GT GCT **C**TC **C**GA **TGT** TCC **C**TA CAT GGG **T**AT T**A**C | DQ886671 |

*__Boldface__ indicates differences in nucleotide sequences. *Bsr*DI restriction sites are underlined.
†Sequences were obtained from GenBank: Bosnia A (*T. pallidum* subsp. *endemicum*), Samoa D (*T. pallidum* subsp. *pertenue*) and syphilis control strain Nichols (*T. pallidum* subsp. *pallidum*).
‡Sequence also confirmed in this study.

the etiology of these clinical cases as *T. pallidum* subspecies *endemicum*.

The boy was administered 600,000 IU of penicillin intramuscularly; 4 weeks later, the ulcer and hoarseness had resolved, and the boy drooled only occasionally. All household members were subsequently treated with penicillin intramuscularly (Table 1).

## Conclusions

We suspect the 1-year-old child acquired her infection through close contact with her 3-year-old brother and other siblings, who themselves acquired endemic syphilis while in the Republic of Senegal. The use of molecular techniques greatly assisted in confirming the clinical diagnosis.

Control programs of the World Health Organization and the United Nations Children's Fund using long-acting penicillin helped reduce the incidence of endemic syphilis and yaws by ≈95% during the 1950s and 1960s. Unfortunately, changes in the administration and delivery of these programs led to an increase in the prevalence of nonvenereal treponematoses during the 1970s (*2*,*5*,*6*). A risk remains for importation of disease into areas where it is not endemic and, subsequently, for local transmission of the etiologic agent.

Diagnosis of imported cases and cases resulting from local transmission is confounded by a lack of experienced clinicians in the diagnosis of nonvenereal treponematoses, and the inability of serologic methods to differentiate these disease entities from venereal syphilis and from each other. Definitive diagnosis is also hampered by widespread unavailability of molecular diagnostics. This deficit necessitates an integrated effort to offer a reproducible reference service to all care providers in a timely and reliable manner to ensure the best clinical outcome, as well as appropriate follow-up for infection control and public health purposes.

Dr Fanella is a pediatric infectious diseases physician at the University of Manitoba. His research interests include methicillin-resistant *Staphylococcus aureus* infections, antibiotic stewardship, blastomycosis, and tropical medicine.

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# Schmallenberg Virus in Calf Born at Term with Porencephaly, Belgium

**To the Editor:** From the end of August through the end of October 2011, a clinical syndrome involving adult cattle and the fetuses of pregnant cows emerged in the border area between the Netherlands and North Rhine-Westphalia, Germany (*1*). The syndrome was characterized by nonspecific clinical signs (fever, decreased milk production), severe diarrhea, and some abortions. A metagenomic analysis was conducted on pooled samples from cattle with acute signs on a farm in the city of Schmallenberg, Germany.

The analysis detected nucleotide sequences homologous to arthropod-borne Akabane, Aino, and Shamonda viruses, all belonging to the family *Bunyaviridae*, genus *Orthobunyavirus*, and Simbu serogroup (*1*). Real-time PCR detected the genomic RNA of the new and emerging virus, tentatively designated Schmallenberg virus (SBV), in the blood of adult cattle, abdominal fluid of a stillborn calf, and brains of lambs born with birth defects on dozens of farms in the Netherlands, Germany, and Belgium. No data are yet available to predict how the emerging virus might affect the cattle industry. We report the case of a 1-week old calf with severe central nervous system (CNS) lesions probably caused by in utero infection with the new virus.

In Belgium in January 2012, a Belgian Blue multiparous cow gave birth to a 45-kg female calf that was morphologically normal but hypertonic and hyperreflexic. Pregnancy had proceeded uneventfully and lasted 9 months and 4 days. Spontaneous reflexes such as sucking, swallowing, micturition, defecation, and crying were completely preserved, but the calf was unable to stand, and its consciousness alternated from mild to severe depression. It was obviously blind and showed ventrolateral strabismus, but the pupils functioned normally. Muscle tone was permanently increased, as indicated by tetanus-like erection of the ears and by a violent but brief startle response to the slightest acoustic or tactile stimulation (Figure). When the calf was placed upright, loss of conscious proprioception was obvious; it maintained its position only a few seconds before collapsing. Altogether, the clinical signs suggested severe dysfunctions of the cerebral cortex, basal ganglia, and mesencephalon. The calf drank from a bottle twice a day for a week, but then was euthanized for humane reasons (infected decubital ulcers).

At necropsy, the cerebellum, brainstem, and diencephalon appeared normal in shape and volume (Figure). However, the cerebral hemispheres were replaced by 2 thin-walled, fluid-filled cysts with some floating islets

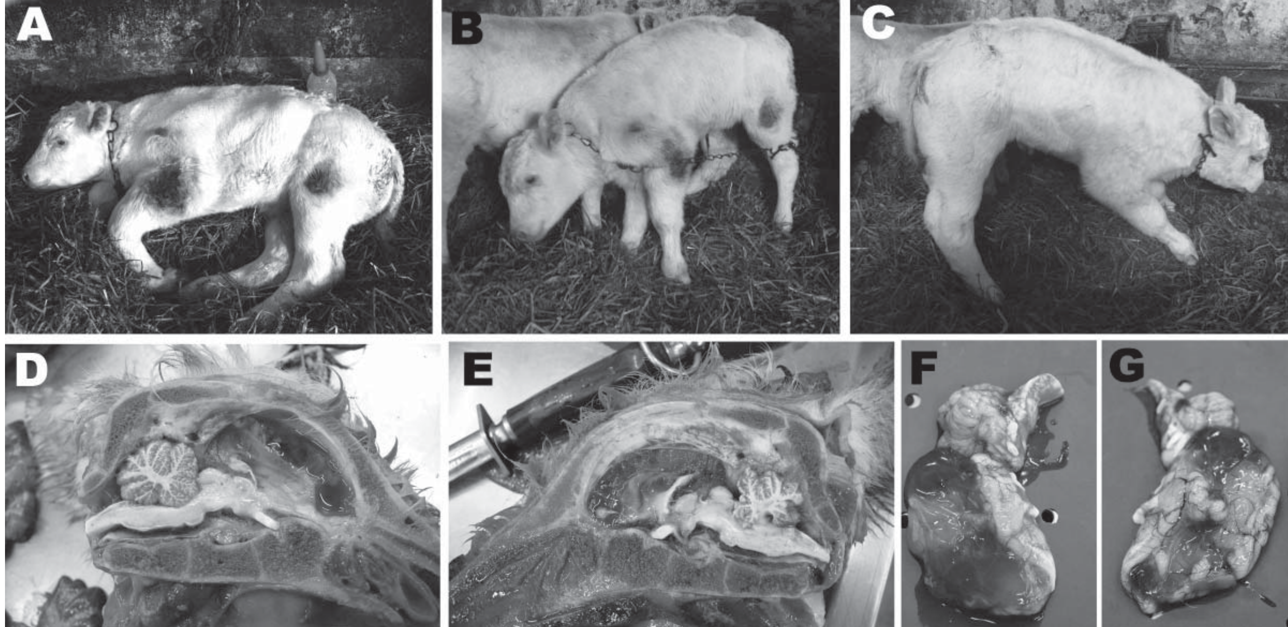


Figure. A 7-day old, female, Schmallenberg virus–positive calf showing severe central nervous system dysfunctions (A–C) and lesions (D–E). A) Spontaneously lying down; B–C) standing with assistance; D–G) porcencephaly, either with the encephalon in place (D–E) or extracted (F–G). The cerebral hemispheres were replaced by 2 thin-walled, fluid-filled cysts (diamonds) with some floating islets and peninsulae corresponding to preserved cortex (stars). The cerebrum was variably preserved, the occipital lobes were totally liquefied, and the outer layers of some parts of the temporal and frontal lobes were irregularly preserved. The cerebellum, brainstem, and diencephalon appeared normal in shape and volume. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/6/12-0104-F.htm).

and peninsulae corresponding to preserved cortex. There was variable preservation of the cerebrum, total liquefaction of occipital lobes, and irregular preservation of the outer layers of some parts of the temporal and frontal lobes. Altogether, the picture was compatible with severe porencephaly or hydranencephaly. The spine showed no sign of scoliosis, and movement of the limb joints was not restricted (i.e., no arthrogryposis).

Samples were removed from the remnants of the cerebrum, diencephalon, and organs (thymus, lung, myocardium, jejunum, ileum, mesenteric lymph node, liver, spleen, kidney, and striated muscle), and 3 independent real-time PCR protocols were conducted to detect genomes of bovine viral diarrhea/mucosal disease virus, bluetongue virus serotype 8, and the novel SBV. Initial retrotranscription of the RNA genomes was followed by quantitative (real-time) PCR. The process was conducted by using our procedures (*2*) and, for SBV, by following the protocol and using recently developed control reagents as described (*1*). The SBV genome was detected in only CNS samples (quantification cycle value 28.8); bovine viral diarrhea/mucosal disease virus and BTV-8 genomes were not detected. The new virus genome load was $1.61 \times 10^4$ copies per gram of cerebrum sample.

Taken together, the above data suggest that, like other Simbu serogroup viruses, the new virus crosses the placenta, contaminates the bovine fetus, infects the fetus' CNS, and causes necrosis and/or developmental arrest of the cerebral cortex. Unlike the viruses mentioned above (*3*,*4*), and provided this case is not an exception, the SBV genome seems to persist in the infected fetus and is detectable after birth by real-time reverse transcription PCR, despite gestation length. Although reliable reagents for detecting seroconversion are temporarily unavailable, the persistence of the new virus in fetal tissue should greatly facilitate the epidemiologic monitoring of the emergence and spread of the new virus.

When calves from experimentally infected dams are infected with the closest phylogenetic relative to SBV, Akabane virus, porencephaly develops during gestational days 62–96 (*5*). If the same is true for the new virus, the above calf was probably infected during June 9–July 13, 2011. Therefore, it is hypothesized that infected arthropods were already circulating in the village of Hamois-in-Condroz (50°24′56″N, 5°8′7″E), which is ≈240 km southwest of Schmallenberg (51°8′42″N, 8°17′18″E), ≈2 months before the emergence of the clinical syndrome that led to the identification of the new virus.

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# Zoonotic Disease Pathogens in Fish Used for Pedicure

**To the Editor:** Doctor fish (*Garra rufa*) are freshwater cyprinid fish that naturally inhabit river basins in central Eurasia. They are widely used in the health and beauty industries in foot spas for ichthyotherapy (Kangal fish therapy or doctor fish therapy) (Figure; online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/pdfs/11-1782-Techapp.pdf) (*1*). During these sessions, patients immerse their feet or their entire bodies in the spas, allowing the fish to feed on dead skin for cosmetic reasons or for control of psoriasis, eczema, and other skin conditions.

A survey during the spring of 2011 identified 279 fish spas in the United Kingdom, and the number has probably increased since then (*1*). The Fish Health Inspectorate of the Centre for Environment, Fisheries & Aquaculture Science estimates that each week 15,000–20,000 *G. rufa* fish are imported from Indonesia and

other countries in Asia into the United Kingdom through London Heathrow Airport (the main border inspection post for the import of live fish). However, ichthyotherapy has now reportedly been banned in several US states and Canada provinces because of sanitary concerns (*1*). In the United Kingdom, a limited number of infections after fish pedicures have been reported (*1*). Unfortunately, little is known about the types of bacteria and other potential pathogens that might be carried by these fish and the potential risks that they might pose to customers or to ornamental and native fish.

On April 12, 2011, the Fish Heath Inspectorate investigated a report of a disease outbreak among 6,000 *G. rufa* fish from Indonesia that had been supplied to UK pedicure spas. Affected fish showed clinical signs of exophthalmia and of hemorrhage around the gills, mouth, and abdomen. More than 95% of the fish died before the remaining fish were euthanized. Histopathologic examinations identified systemic bacterial infections with small gram-positive cocci, mostly in the kidneys, spleen, and liver. Bacterial isolates cultured from affected fish were identified as *Streptococcus agalactiae* (group B *Streptococcus*) according to a combination of biochemical test results (API Strep; bioMérieux, Marcy l'Étoile, France), Lancefield grouping with serotype B (Oxoid Limited, Basingstoke, UK), and molecular (partial 16S rRNA gene sequencing) testing methods.

Multilocus sequence typing of a representative isolate (11013; online Technical Appendix Table) (*2*) indicated that it was a sequence type (ST) 261 *S. agalactiae* strain (http://pubmlst.org/sagalactiae). This same ST261 profile was first identified in an isolate (ATCC 51487) from a diseased tilapia in Israel (*3*). The clinical appearance of the disease and the diagnostic results suggested that *S. agalactiae* was the causative agent of the fish illness and deaths.

To determine whether *S. agalactiae* and other bacterial pathogens might be carried more widely by these fish, from May 5, 2011, through June 30, 2011, the Fish Health Inspectorate of the Centre for Environment, Fisheries & Aquaculture Science visited Heathrow Airport 5 times to intercept and sample consignments of *G. rufa* from Indonesia. A taxonomically diverse range of bacteria were identified (online Technical Appendix Table, Figure 2), including a variety of human pathogens capable of causing invasive soft tissue infections. These pathogens included *Aeromonas* spp. (*4*), potentially pathogenic clinical-type *Vibrio vulnificus* isolates (online Technical Appendix Figure 2) (*5*), non–serotype O1 or O139 cholera toxin–negative *V. cholerae* isolates (online Technical Appendix Figure 2) (*6*), *Mycobacteria* (*7*), and *S. agalactiae* (*3,8*). Isolates were resistant to a variety of antimicrobial drugs, including tetracyclines, fluoroquinolones, and aminoglycosides (online Technical Appendix Table). Other studies have also reported high levels of multidrug resistance in bacteria associated with imported ornamental fish (*9*).

Water is a well-recognized source of bacterial skin infections in humans. *V. vulnificus* can cause wound infections and primary septicemia, resulting in high mortality rates, especially among persons with predisposing risk conditions (e.g., liver disease, diabetes, or impaired immune function) (*5*). *S. agalactiae* is a common cause of skin and soft tissue infections, especially in older adults and those with chronic diseases such as diabetes mellitus (*8*). Although *S. agalactiae* ST261 is not considered to be one of the genotypes typically associated with invasive disease in humans (*3*), a fish-adapted strain could eventually take advantage of the opportunity afforded by repeated exposure and thereby also affect

Figure. Doctor fish surrounding foot during ichthyotherapy.

humans. Additionally, *Mycobacteria* spp. can occasionally cause disease in humans through contact with fish (*M. marinum*), and pedicure treatments have previously been associated with *M. fortuitum* infections (*10*).

Recently, the risks associated with exposure to *G. rufa* fish were reported to be low (*1*). To date, there are only a limited number of reports of patients who might have been infected by this exposure route (*1*). However, our study raises some concerns over the extent that these fish, or their transport water, might harbor potential zoonotic disease pathogens of clinical relevance. In particular, patients with underlying conditions (such as diabetes mellitus or immunosuppression) should be discouraged from undertaking such treatments, especially if they have obvious breaks in the skin or abrasions. This risk can probably be reduced by use of certified disease-free fish reared in controlled facilities under high standards of husbandry and welfare.

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## *Rickettsia conorii* Indian Tick Typhus Strain and *R. slovaca* in Humans, Sicily

**To the Editor:** Rickettsiae are vector-borne pathogens that affect humans and animals worldwide (*1*). Pathogens in the *Rickettsia conorii* complex are known to cause Mediterranean spotted fever (MSF) (*R. conorii* Malish strain), Astrakhan fever (*R. conorii* Astrakhan strain), Israeli spotted fever (*R. conorii* Israeli spotted fever strain), and Indian tick typhus (*R. conorii* Indian tick typhus strain) in the Mediterranean basin and Africa, southern Russia, the Middle East, and India and Pakistan, respectively (*2*). These rickettsioses share some clinical features, such as febrile illness and generalized cutaneous rash, and are transmitted to humans by *Rhipicephalus* spp. ticks (*2*).

MSF is endemic to Sicily (Italy); fatal cases occur each year, and the prevalence of *R. conorii* in dogs is high (*3–6*). Recently, *R. conorii* Malish strain and *R. conorii* Israeli spotted fever strain were confirmed in humans in Sicily in whom MSF was diagnosed (*4*), which suggests that other *R. conorii* strains might be present and diagnosed as causing MSF. The rickettsiae within the *R. conorii* complex, which are relevant for the study of bacterial evolution and epidemiology, can be properly identified only by appropriate genetic analyses.

We analyzed 15 blood and 19 inoculation eschar samples collected during 2005–2009 from 31 patients in Palermo Province and 2 in Catania Province, none of whom had recently traveled. None were severely ill, but all 33 had clinical manifestations and laboratory results compatible with MSF: 1-week incubation after tick bite, fever, headache, myalgia, papulonodular rash that started on the upper limbs and spread centripetally with or without tache noire, and detection of antibody titers ≥180 to *R. conorii* by indirect immunofluorescence antibody test (bioMérieux, Marcy L'Etoile, France).

Total DNA was extracted by using the GeneElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, Milan, Italy) and used to analyze *Rickettsia* spp. sequences by PCR, cloning, and sequence analysis of the amplicons. At least 3 clones were sequenced for each amplicon. Genes targeted by PCR included ATP synthase α subunit (*atpA*) (*7*), heat-shock protein 70 (*dnaK*) (*7*), outer membrane protein A (*ompA*) (primers Rr190.70p and 190–701 [*8*]), outer membrane protein B (*ompB*) (primers rompBSFGIF and rompBSFG/TGIR [*9*]), citrate synthase (*gltA*) (*2*), and 17-kDa protein (primers TZ15–19 and TZ16–20 [*6*]). Nucleotide sequence identity to reference strains (*2*), multilocus analysis by *atpA–dnaK–ompA–ompB–gltA–17-kDa* and *ompA–ompB* sequences and in silico *Pst*I-*Rsa*I restriction analysis of *ompA* sequences (*8*) were used to characterize *Rickettsia* spp. and *R. conorii* strains.

Results for 15 (45%) patients were positive for *Rickettsia* spp. Thirteen isolates were confirmed as *R. conorii* Malish strain (identification [ID] nos. 44, 45, 47, 49, 54, 55, 57, 59, 61, 66, 68, 92, 112) and 1 each as *R. conorii* Indian tick typhus strain (ID no. 58) and *R. slovaca* (ID no. 50). *R. slovaca* DNA was also found in a *Dermacentor marginatus* tick removed from the patient who had confirmed *R. slovaca* infection. *R. conorii* Malish strains showed 99.9%–100%, 100%, 100%, 98.7%–100%, 100%, and 97.8%–100% pairwise nt sequence identity to reference strain Malish 7 (AE006914) *atpA*, *dnaK*, *ompA*, *ompB*, *gltA*, and 17-kDa protein, respectively.

The *R. conorii* Indian tick typhus strain showed 100%, 100%, 99.4%, 100%, 100%, and 99.9% pairwise nt sequence identity to *R. conorii* strain Malish 7 (AE006914) *atpA*, *dnaK*, 17-kDa protein, and *R. conorii* Indian tick typhus reference strain *ompA* (U43794), *ompB* (AF123726), and *gltA* (U59730), respectively. The *R. slovaca* strain showed 99.4%, 97.8%, 100%, 93.7%, 99.7%, and 99.4% pairwise nt sequence identity to *R. slovaca atpA* (AY124734), *dnaK* (DQ821824), *ompA* (HM149286), *ompB* (HQ232242), *gltA* (AY129301), and *R. conorii* strain Malish 7 (AE006914) 17-kDa protein, respectively. The sequences were deposited in GeneBank under accession nos. JN182782–JN182804.

Multilocus sequence analysis (Figure, panel A) and in silico *Pst*I-*Rsa*I restriction analysis of *ompA* sequences also confirmed the identity

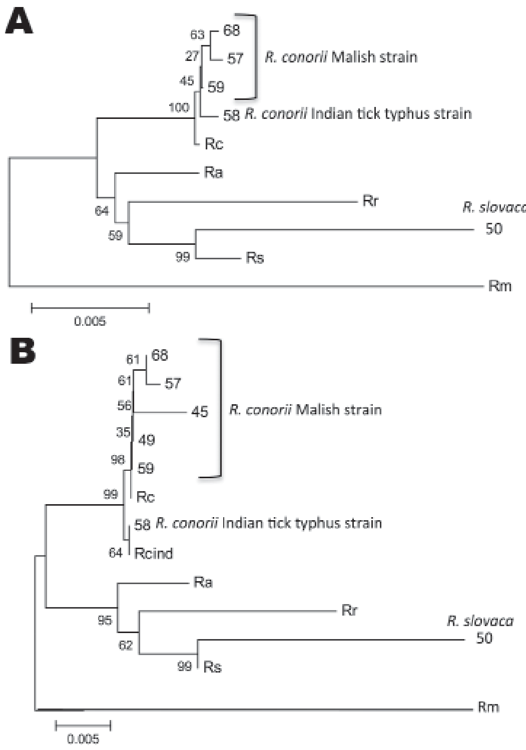


Figure. Multilocus sequence analysis of *Rickettsia* spp. Evolutionary history was inferred by using the neighbor-joining method for ATP synthase α subunit (*atpA*)–heat shock protein 70 (*dnaK*)–outer membrane protein A (*ompA*)–*ompB*–citrate synthase (*gltA*)–17-kDa (A) and *ompA*–*ompB* sequences (B). The optimal tree with the sum of branch length = 0.06205323 (A) and 0.11097561 (B) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic relationship. Evolutionary distances were computed by using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA5 (www.megasoftware.net). Identification numbers of strains detected are shown with the species/strain name next to them. Rc, *R. conorii* strain Malish 7; Ra, *R. africae* strain ESF-5; Rr, *R. rickettsii* strain Iowa; Rs, *R. slovaca*; Rm, *R. massiliae* strain MTU5; Rcind, *R. conorii* Indian tick typhus strain.

of the *Rickettsia* spp. we identified. As shown (*2*), multilocus analysis with *ompA–ompB* sequences was highly informative about the phylogenetic relationship between *Rickettsia* spp. and *R. conorii* strains (Figure, panel B).

In Sicily, *R. conorii* Malish strain has been characterized in MSF patients (*4*), and *R. slovaca* DNA was identified in ixodid ticks (*5*). However, to our knowledge, *R. slovaca* in humans in Sicily and *R. conorii* Indian tick typhus strain infection in Sicily and Europe have not been reported. The only previous report outside India and Pakistan was documented in a traveler with severe clinical manifestations in France (*10*). Differences were not observed between *R. conorii* Indian tick typhus strain and *R. slovaca*–infected patients. Both patients had similar clinical symptoms compatible with MSF; in both, only IgM for rickettsiae was detected at hospital admission, but IgM and IgG were detected during convalescence. Tache noire were detected in the neck and right arm of patients with *R. conorii* Indian tick typhus strain and *R. slovaca*, respectively.

These results demonstrated that new rickettsiae, such as *R. conorii* Indian tick typhus strain, of public health relevance are emerging in Europe. The widespread distribution of tick vectors in Europe and the transtadial and transovarial transmission of the pathogen in ticks might favor transmission to humans.

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# Detection of European Strain of *Echinococcus multilocularis* in North America

**To the Editor:** In 2009, an alveolar hydatid cyst, the intermediate stage of the cestode *Echinococcus multilocularis*, was detected in the liver of a dog from Quesnel, British Columbia (BC), Canada (*1*), 600 km west of the nearest known record of this parasite in central North America (Figure). Alveolar hydatid cysts normally occur in rodent intermediate hosts. However, humans can serve as aberrant intermediate hosts; cysts generally originate in the liver and, in about one third of cases, metastasize throughout the body (*2*). Detection of the larval stage of this pathogen in an unusual host in a new geographic region required application of multiple molecular epidemi-

ologic techniques to determine if this was range expansion of a native strain or introduction of a new strain of veterinary and public health concern.

Alveolar hydatid cyst material was surgically excised from the dog, frozen, and shipped to the Western College of Veterinary Medicine in Saskatoon, Saskatchewan. DNA was extracted by using the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN, Inc., Valencia, CA, USA). PCR was performed by using primers for 4 mitochondrial loci: NADH dehydrogenase subunit 1 (*nad1*) and 2 (*nad2*), cytochrome b (*cob*), and cytochrome c oxidase subunit 1 (*cox1*) (*3*,*4*). Sequence of a 488-bp region of the *nad1* gene (GenBank accession no. JF751034) was 99%–100% identical to *E. multilocularis* sequences from Asia (AY389984) and Europe (AB668376). Sequence data for 91 of 112 positions at the *cox1* (partial), *cob1*, and *nad2* genes (GenBank accession nos. JF751033, JF751035, and JF751036) grouped with haplotypes from Europe (*4*); 2 nucleotide differences from the E4 haplotype in foxes in France and Belgium were found, and 1 additional nucleotide difference (position 663 in *cob1*) did not correspond to any of the haplotypes defined previously.

Two independent subsamples of cyst material were fixed in 70% ethanol and shipped to the University of Regina, Regina, Saskatchewan, for PCR using primers targeting microsatellite loci EmsB and NAK 1 (*5*,*6*). PCR products were sized with single-basepair resolution by using capillary electrophoresis on a DNA sequencer (Genome Lab GeXP; Beckman-Coulter, Fullerton, CA, USA). Peaks that had <15% of the amplitude of the highest peak were excluded from analysis. EmsB electrophoregrams from the 2 samples of cyst material were identical and displayed 10 peaks spanning 220–238 bp. Visually, the EmsB profile from the BC dog sample most closely matched European profile H from foxes in west-central Europe (*5*). The BC dog sample was homozygous for the 198-bp allele at NAK 1, a genotype found in a variety of locations in Europe and Japan but not in North America, where the dominant genotype appears to be homozygous 192 (*5*).

Mitochondrial and microsatellite characterization showed that the genotype of *E. multilocularis* found in the BC dog was most similar to those described from west-central Europe. If the BC report represented a westward range expansion of a native North American strain of *E. multilocularis*, it would most likely be the N2 strain, established in the North Central Region, which includes the 3 Canadian prairie provinces and 12 contiguous north central American states (*4*) (Figure). This case demonstrates the utility of molecular epidemiology for detecting incursion of foreign pathogens and tracing their origins, as well as the feasibility of using animal sentinels to detect the introduction of a disease of public health concern into a new area.

Because the dog had never left BC and cestode eggs were not de-

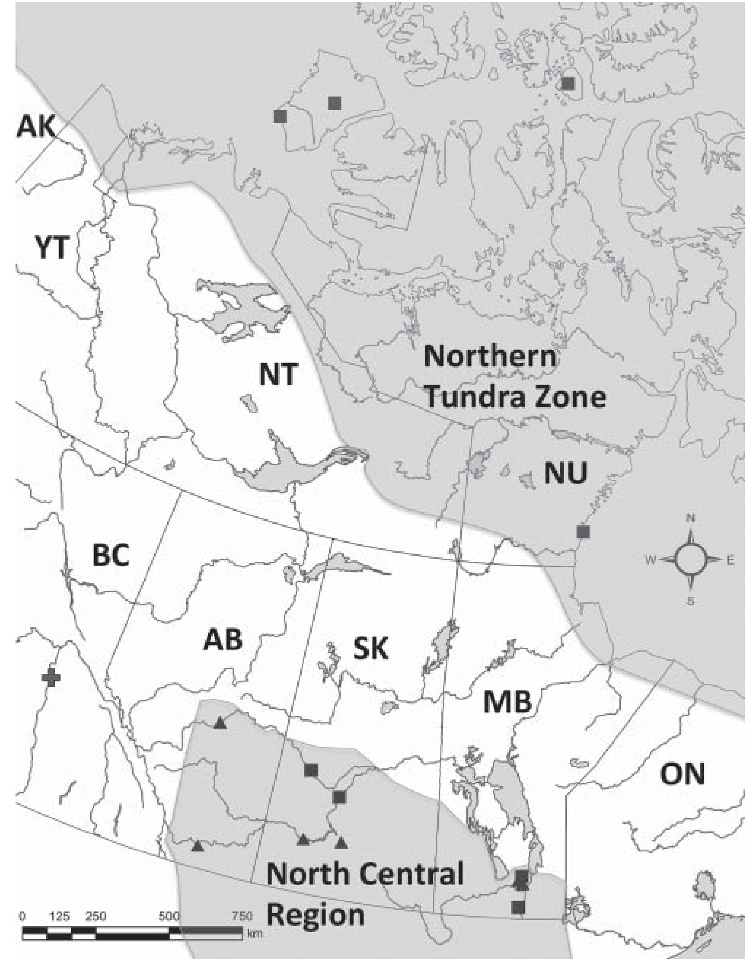


Figure. Location where European-type strain of *Echinococcus multilocularis* (plus sign) was detected in this study in British Columbia (BC) and previous reports of *E. multilocularis* parasites in 8 definitive (squares) and 6 intermediate (triangles) hosts in Canada. Gray shading indicates currently accepted distribution of *E. multilocularis* in North America. The North Central Region includes southern portions of the 3 Canadian prairie provinces (Alberta [AB], Saskatchewan [SK], and Manitoba [MB]) and 12 contiguous US states (not shown). The western portion of the Northern Tundra Zone is based roughly on the established distribution of Arctic fox in Alaska (AK), the Yukon Territory (YT), Northwest Territories (NT), Nunavut (NU), northern MB, and northern Ontario (ON).

tected from a single fecal sample examined on microscopy (*1*), infection most likely resulted from consumption of infective eggs in the feces of a carnivore-definitive host. This host could have been a translocated domestic dog, thought to be the mechanism of recent introduction of *E. multilocularis* parasites into Sweden (*7*). It is also possible that a European strain of the parasite was introduced into North America in the last century, when red fox from France and Scandinavia were introduced (*8*).

The possible establishment of a European strain in North American wildlife, with spillover into domestic dogs, may have implications for public health and require increased vigilance by medical and veterinary personnel in the newly endemic region. Compared with native North American strains, European strains of *E. multilocularis* appear to have greater potential to cause alveolar hydatid disease (AHD) in humans. These strains are emerging worldwide (increasing in both prevalence and distribution) as a result of changes in landscape, climate, and wildlife–human interfaces (*2*,*9*,*10*). In Europe, human AHD can be fatal (definite or probable cause of death in 23.5% of 119 recent cases) and has low cure rates (5% of 408 recent cases) (*2*). As of 2000, in Europe and Asia, the estimated cost per case of AHD was US $100,000–$300,000 (*9*). Therefore, better understanding of the distribution, genetic diversity, and pathogenicity of strains of *E. multilocularis* is needed to assess risks and mitigate costs for public and veterinary health, as well as to provide evidence for the regulation and screening of imported domestic animals and translocated wildlife.

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## Recognition and Diagnosis of *Cryptococcus gattii* Infections in the United States

**To the Editor:** An outbreak of *Cryptococcus gattii* cryptococcosis has been ongoing in the US Pacific Northwest (PNW) since 1999 (*1*–*3*). In contrast to *C. neoformans* infections, which typically cause meningitis in HIV-infected persons, outbreak-associated *C. gattii* infections occur primarily in persons without HIV and often cause pneumonia (*1*–*3*). Sporadic, nonoutbreak-associated *C. gattii* infections often cause meningitis and have been reported outside the PNW (*1*–*4*). The prevalence of both types of *C. gattii* infection in the United States is unknown because diagnostic practices and awareness vary among physicians.

Some reports indicate that patients with *C. gattii* infections may respond to treatment more slowly and relapse more frequently than patients with *C. neoformans* infections and, thus, may require more aggressive clinical management (*5*–*8*). Therefore, differentiation of *C. gattii* from *C. neoformans* infections may be necessary for optimal patient

management. However, cryptococcal infections are often diagnosed by antigen testing, which cannot distinguish between *C. gattii* and *C. neoformans,* and differential agar necessary to distinguish species in culture (*9*) is not uniformly used in clinical laboratories. In addition to possible missed diagnoses caused by the atypical manifestation of outbreak-associated *C. gattii*, outbreak-associated and sporadic *C. gattii* infections in the United States are likely being misdiagnosed as *C. neoformans* infections.

We conducted a survey of US infectious disease physicians to better understand the clinical approach to diagnosing cryptococcal infections, the relative regional frequency of *C. gattii*, and the capacity of clinical laboratories to differentiate cryptococcal species. To survey physicians, we used the Emerging Infections Network (EIN), a sentinel public health surveillance system of infectious disease clinicians that is supported by the Centers for Disease Control and Prevention and sponsored by the Infectious Diseases Society of America (*10*). During February–March 2011, web-based surveys were distributed by email or fax to the 1,342 EIN members, of whom 792 (59%) responded.

Analysis was restricted to 286 (36%) respondents (representing 43 states) who treated a cryptococcosis patient during the past year. We compared answers from respondents in the 4 US census regions (Table; online Appendix Figure, wwwnc.cdc.gov/EID/article/18/1/11-1228-FA1.htm). Results were analyzed by using SAS version 9.2 (SAS Institute Inc., Cary, NC).

The approximate number of reported physician consults for cryptococcosis was similar among respondents from all regions (Table). More respondents from the West (40%), compared with the South (21%), Midwest (22%), and Northeast (19%), reported that >25% of their cryptococcosis patients had pneumonia; this finding may reflect the higher prevalence of outbreak-associated *C. gattii* infections in the West (*1–3*). The percentage of respondents who treated

Table. Physician responses, by US region, to a survey about cryptococcosis, February–March 2011*

| Question and responses | No. (%) responding physicians† | | | | |
|---|---|---|---|---|---|
| | Overall, n = 286 | Northeast, n = 48 | Midwest, n = 63 | South, n = 113 | West, n = 62 |
| No. patients with cryptococcosis seen during the past year | | | | | |
| 1–4 | 218 (76) | 41 (85) | 55 (87) | 71 (63) | 51 (82) |
| 5–8 | 49 (17) | 6 (13) | 7 (11) | 29 (26) | 7 (11) |
| 9–12 | 12 (4) | 1 (2) | 1 (2) | 8 (7) | 3 (5) |
| >12 | 7 (2) | 0 | 0 | 5 (4) | 1 (2) |
| Percentage of patients with cryptococcal pneumonia, with or without meningitis | | | | | |
| 0–25 | 213 (75) | 39 (81) | 49 (78) | 89 (79) | 36 (59) |
| 26–50 | 33 (12) | 1 (2) | 6 (10) | 13 (12) | 13 (21) |
| 51–75 | 8 (3) | 1 (2) | 1 (2) | 4 (4) | 2 (3) |
| 76–100 | 31 (11) | 7 (15) | 7 (11) | 7 (6) | 10 (16) |
| Method used to obtain a diagnosis of cryptococcosis (all that apply) | | | | | |
| Cryptococcal antigen test | 272 (95) | 48 (100) | 58 (92) | 110 (97) | 56 (90) |
| Microscopy | 95 (33) | 16 (33) | 13 (21) | 42 (37) | 24 (39) |
| Culture | 210 (73) | 33 (69) | 50 (79) | 82 (73) | 45 (73) |
| Histopathology | 75 (26) | 10 (21) | 10 (16) | 31 (27) | 24 (39) |
| Any combination of tests that does not include culture | 76 (27) | 15 (31) | 13 (21) | 31 (27) | 17 (27) |
| Clinical laboratory routinely or on request can differentiate *Cryptococcus neoformans* from *C. gattii*‡ | 131 (66) | 20 (67) | 28 (68) | 48 (64) | 35 (66) |
| Percentage of cryptococcal infection cases in HIV-uninfected patients | | | | | |
| 0–25 | 154 (54) | 32 (68) | 26 (41) | 70 (62) | 26 (44) |
| 26–50 | 48 (17) | 5 (11) | 15 (24) | 16 (14) | 12 (20) |
| 51–75 | 32 (11) | 3 (6) | 9 (14) | 11 (10) | 9 (15) |
| 76–100 | 51 (18) | 7 (15) | 13 (21) | 16 (14) | 12 (20) |
| Diagnosed cryptococcal infections in HIV-uninfected patients with no known risk factors for infection during past 5 y | 78 (27) | 6 (13) | 13 (21) | 26 (23) | 33 (53) |
| Considers species of *Cryptococcus* as a factor of interest in diagnosis or when treating a patient | 179 (63) | 22 (46) | 36 (57) | 71 (63) | 50 (81) |
| Considered *C. gattii* infection as a differential diagnosis for pneumonia in a person from the US Pacific Northwest | 153 (54) | 19 (40) | 29 (46) | 63 (56) | 42 (68) |
| Ever treated or consulted on a patient known to have *C. gattii* infection | 38 (13) | 5 (10) | 3 (5) | 3 (3) | 27 (44) |

*The survey was conducted by the Emerging Infections Network among physician members; responses are from providers who had seen any patients with cryptococcosis during the preceding year. Region is defined by the 4 census regions: Northeast (Connecticut, Maine, Massachusetts, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, Vermont), Midwest (Indiana, Illinois, Iowa, Kansas, Michigan, Minnesota, Missouri, Nebraska, North Dakota, Ohio, South Dakota, Wisconsin), South (Alabama, Arkansas, Delaware, District of Columbia, Florida, Georgia, Kentucky, Louisiana, Maryland, Mississippi, North Carolina, Oklahoma, South Carolina, Tennessee, Texas, Virginia, West Virginia), West (Alaska, Arizona, California, Colorado, Hawaii, Idaho, Montana, New Mexico, Nevada, Oregon, Washington, Wyoming).
†Not all respondents answered all questions.
‡Excludes “don’t know” responses.

cryptococcosis patients without known risk factors for infection (including HIV) during the past 5 years was also higher in the West (53%) compared with other regions (Table).

Most (93%) respondents reported that they were aware of the *C. gattii* outbreak. However, only 63% of respondents consider *Cryptococcus* species a factor of interest during diagnosis or treatment, and 54% would consider *C. gattii* as a differential diagnosis for pneumonia in a patient from the PNW. Although awareness of *C. gattii* appears high, recognition of infection may be delayed when diagnostic plans do not include species identification.

Of the respondents, 94% reported that they most often use the cryptococcal antigen test for diagnosis, although 73% of respondents report that they commonly request a culture. Furthermore, 76 (27%) of respondents report using a combination of tests (cryptococcal antigen, microscopy, histopathology) that does not include culture. Tests that do not differentiate between cryptococcal species represent missed opportunities for diagnosis of *C. gattii* infections. When respondents were asked if their clinical laboratory could differentiate *C. neoformans* from *C. gattii* isolates, 131 (46%) responded "yes, either routinely or when requested"; 68 (24%) responded "no"; 87 (30%) did not know. When we excluded respondents who did not know, only 66% of respondents from the West indicated that their laboratory could differentiate species. This finding is concerning because outbreak-associated *C. gattii* is clearly endemic to the region. A better understanding of which laboratories perform this service and which send specimens to a reference laboratory will help identify where additional capacity is needed.

A lower percentage of respondents from the Northeast (10%), Midwest (5%), and South (3%), compared with those from the West (44%), reported having ever consulted on a case of *C. gattii* infection. This may reflect a low incidence of *C. gattii* infections in these regions, or it may be a result of decreased clinical suspicion for *C. gattii* infections outside the PNW.

Results from this study suggest that although most EIN members are aware of *C. gattii* and the ongoing outbreak in the PNW, missed opportunities for diagnosis still exist. To understand the true incidence of *C. gattii* inside and outside the PNW, vigilance among physicians nationwide is necessary. Clinicians and laboratorians should be aware of the need to obtain specimens for culture and of the need to develop methods to differentiate cryptococcal species. An accurate diagnosis of cryptococcosis cases in the United States will lead to a better understanding of the epidemiology and incidence of *C. gattii* in this country and may result in improved treatment.

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# Coccidioidal Endophthalmitis in Immunocompetent Person, California, USA

**To the Editor:** In the United States, dimorphic fungi of the species *Coccidioides* are endemic to California (particularly the Central Valley), southern Arizona, southern New Mexico, and western Texas. Although there are a relatively large number of coccidioidomycosis cases in the United States (≈150,000/year), intraocular coccidioidomycosis is uncommon (*1*,*2*). We report a case of coccidioidal endophthalmitis in an immunocompetent person.

In October 2010, a 55-year-old white man in Santa Clarita, California, had severe pneumonia, drenching sweats, and an associated 25-pound weight loss. Three weeks later, when his symptoms had nearly resolved, the man reported having scratched his left eye with his eyeglasses and subsequent development of increasing redness, pain, and progressive vision loss (from 20/10 to 20/60 without correction).

In November 2010, the man sought the care of an ophthalmologist, who noted panuveitis of the left eye. Laboratory testing was performed: the erythrocyte sedimentation rate was 49 mm/h (reference 0–22 mm/h), and test results were negative for human leukocyte antigen B27, angiotensin-converting enzyme, rapid plasma reagin, and antinuclear antibody. The patient was started on topical corticosteroids and escalated to high-dose prednisone soon thereafter without improvement. Pain continued to increase in his left eye, and visual acuity declined to hand motion only.

Thus, in February 2011, the patient was referred to our institution, where an ocular ultrasound showed vitreous opacities (Figure). He underwent vitrectomy with intravitreal injection of empiric antimicrobial drugs, including voriconazole. Aqueous fluid obtained intraoperatively grew mold, and the patient was admitted to the hospital for systemic antifungal therapy.

The patient's history was unremarkable except for avid mountain biking in the Central Valley of California. His physical examination was notable for left visual acuity limited to hand motion only, limited extraocular movement, conjunctival injection, and hypopyon. His HIV test result was negative. Computed tomography (CT) scanning of the chest showed micronodules in the right upper lobe, suggesting previous pulmonary coccidioidal infection. Intravenous voriconazole (4 mg/kg every 12 hours) was administered along with daily intravitreal injections of voriconazole while the patient was hospitalized. Results for coccidioidal antibody testing were positive by enzyme immunoassay and immunodiffusion but negative by serum complement fixation. Nucleic acid hybridization testing of aqueous fluid cultures identified *Coccidioides* spp. Results of a CT brain scan, lumbar puncture, and bone scan were normal.

After 1 week of hospitalization, the patient was discharged on oral voriconazole (4 mg/kg 2×/day). Because of transaminitis, the patient was transitioned to fluconazole (800 mg/day) 4 weeks later. He underwent 13 subsequent intravitreal injections of amphotericin and voriconazole. Eleven months after discharge, the patient's best-corrected visual acuity was 20/25, and his ocular media were clear and without any lesions.

Coccidioidomycosis often goes undetected because up to 60% of affected patients are asymptomatic (*3*). When signs and symptoms are present, they vary from subclinical infection to acute pneumonia to disseminated disease (*3*). The rate of extrapulmonary complications is estimated at 0.5% of infections in white persons, but such complications may occur in

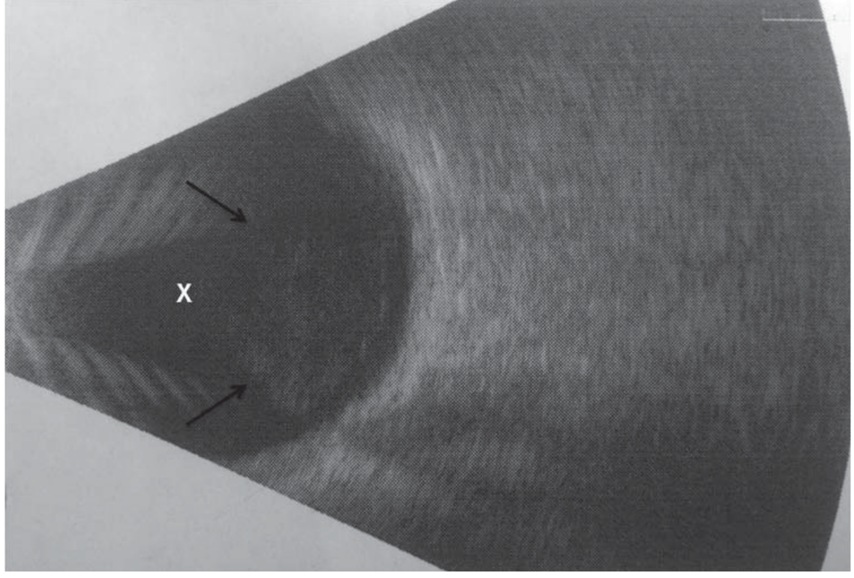


Figure. Ocular ultrasound demonstrating hyperechoic, punctate opacities (arrows) within the vitreous chamber (X) of a patient with coccidioidomycosis, California, USA.

30%–50% of immunosuppressed patients with coccidioidomycosis (*3*). Disseminated coccidioidomycosis typically involves the skin, meninges, or bone (*3*); however, intraocular involvement has also been described (*1*). A review of the literature shows 25 reported cases of intraocular coccidioidomycosis. When present, intraocular involvement is associated with serious consequences, frequently leading to eye enucleation; 1 case series described eventual enucleation in 50% of reported patients who did not die from disseminated coccidioidal infection (*2*).

For the patient in our report, in the setting of reported trauma and negative metastatic work-up results, it is unclear whether ocular disease resulted independently as an exogenous infection or from endogenous lymphatic and/or hematogenous spread from the patient's lung. Diagnosis of coccidioidal endophthalmitis can be difficult, often relying on serum or nonocular tissue evaluation (*4*). Intraocular coccidioidal involvement usually occurs with widespread infection (*5*). Thus, even with apparent isolated ocular findings, evaluation for disseminated disease is warranted, including a careful history and physical examination, CT chest scan, bone scan, intracranial imaging, and lumbar puncture. Evaluation for immunosuppression, including HIV status, is warranted.

The optimal systemic antifungal therapy for intraocular coccidioidal infection is unclear, although fluconazole is the drug of choice for extrapulmonary coccidioidomycosis, including meningitis (*3*). Fluconazole has good ocular penetration; however, voriconazole also achieves excellent intraocular levels (*6*) at lower 90% minimum inhibitory concentration levels (*7*). Furthermore, Gabrielian and Hariprasad (*8*) described an immunocompetent patient with treated and stable nonocular disseminated coccidioidomycosis who showed development of new vitritis and choroiditis 8 weeks into high-dose fluconazole therapy; his intraocular disease resolved within 2–4 weeks of transition to voriconazole.

The patient in our report received systemic voriconazole for 4 weeks plus repeated intravitreal voriconazole injections on follow-up. It is possible that this initial therapy had an effect on his positive outcome and the avoidance of eye enucleation. The optimal length of therapy is unclear; however, this patient will receive prolonged treatment (≥1 year) with high-dose fluconazole, followed by a slow taper guided by serologic testing and regular ophthalmologic examination. Future research should evaluate which antifungal therapy is superior and the appropriate duration of treatment.

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# Human MRSA Isolates with Novel Genetic Homolog, Germany

**To the Editor:** Methicillin-resistant *Staphylococcus aureus* (MRSA) represents a major cause of hospital-, community- and livestock-acquired infections that are increasingly difficult to manage (*1–3*). Detection and identification of MRSA by culture and nucleic acid–based methods is challenged by heterogeneous penicillin-binding protein 2a (PBP2a) expression and variability of the staphylococcal cassette chromosome (SCC*mec*) elements. Recently, a new SCC*mec* element (XI) carried in bovine and human isolates was described (*4,5*). This SCC*mec* element contains a novel *mecA* homolog, designated $mecA_{LGA251}$, that is not detectable by usual *mecA*-specific PCR approaches and PBP2a agglutination tests. García-Álvarez et al. reported this novel *mecA* homolog exhibited 70%

identity at DNA level to the *mecA* gene, and suggested these strains were transmitted from livestock to humans (*4*).

To search for isolates possessing the novel $mecA_{LAG251}$, we screened *S. aureus* databases for those entries describing oxacillin/cefoxitin-resistant phenotypes that were negative for *mecA* by PCR (*6*) or harbored *S. aureus* protein A gene (*spa*) types known to be associated with the occurrence of $mecA_{LGA251}$ (*4*,*5*). The databases of the University Hospital Münster contain *S. aureus spa* typing results of *S. aureus* isolates obtained from hospital admission screenings and specimens from patients treated at University Hospital Münster. Moreover, they include isolates derived from human and animal subjects, respectively, of 2 cross-border projects between the Netherlands and Germany: MRSA-net EUREGIO Twente/Münsterland and SafeGuard MRSA vet-net (*2*,*7*).

The presence of $mecA_{LGA251}$ was verified by using a specific PCR that applied newly designed primers: mecAL1 (5′-AGC TGG CCA TCC CTT TAT TT-3′) and mecAL2 (5′-CTG GCA TAT GGA GAA GAA GAA A-3′), derived from the sequence of *S. aureus* LGA251 provided by M. Holden (Wellcome Trust Sanger Institute, Hinxton, UK; accession no. FR821779). The sensitivities and specificities of primers were checked by applying *S. aureus* and other staphylococcal isolates of different clonal backgrounds (*8*,*9*). Positive PCR products were sequenced to confirm identification of $mecA_{LGA251}$; the isolates were then characterized by typing the SCC*mec* region with specific primers for *mecR1*, *mecI*, *blaZ*, *ccrA*, and *ccrB* related to type XI SCC*mec* as described by García-Álvarez et al. (*4*). Identified isolates were tested for PBP2a by using a latex agglutination assay (Oxoid Deutschland GmbH, Wesel, Germany). We used Etest (bioMérieux SA, Marcy-l'Étoile, France) for antibacterial agent susceptibility testing of β-lactams and other antibacterial agents.

We report on 16 (clinically derived, n = 14; ovine origin, n = 2) oxacillin/cefoxitin-resistant *S. aureus* isolates possessing the recently described $mecA_{LGA251}$ isolate, but lacking the classical *mecA* gene currently defining classic MRSA (Table). The isolates belong to *spa* types t843, t978, t1535, t1773, and t7189. Concurring with the findings in the United Kingdom and Denmark, we found t843 to be the most prevalent *spa* type. Results of the PBP2a latex agglutination assay were negative for all isolates except for 1 (no. 14), which was indeterminable. García-Álvarez et al. described negative results for all tested isolates (*4*); Shore et al. reported inconsistent results with this test (*5*).

According to the Clinical and Laboratory Standards Institute MIC interpretative standards for staphylococci (*10*), antibacterial agent susceptibility testing revealed resistance to benzylpenicillin and oxacillin/cefoxitin for all isolates. All isolates were shown to produce β-lactamases. Apart from the general categorization of oxacillin/cefoxitin-resistant isolates as resistant to all β-lactams, the MICs of drugs for all isolates included were read as susceptible for imipenem (MIC for 90% of strains tested 0.5 µg/mL) as well as for the anti-MRSA cephalosporin ceftobiprole (MIC for 90% of strains tested 1 µg/mL applying provisional breakpoint ≤4 µg/mL). A large range of MICs were observed for classic cephalosporins, ranging from those isolates categorized as susceptible

Table. Description of $mecA_{LGA251}$-positive isolates regarding their *spa* type, ability to grow on selective MRSA media, PBP2a agglutination, *mec* gene possession, and SCC*mec*-type*

| Isolate no. and origin | Characteristics | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Year of isolation | Specimen | *spa* type | Growth on selective MRSA medium† | PBP2a agglutination | Presence of | | |
| | | | | | | *mecA* | $mecA_{LGA251}$ | SCC*mec*XI |
| Human | | | | | | | | |
| 1 | 2010 | Nasal swab | t843 | + | – | – | + | + |
| 2 | 2010 | Wound | t843 | + | – | – | + | + |
| 3 | 2010 | Wound | t843 | + | – | – | + | + |
| 4 | 2010 | Nasal swab | t843 | + | – | – | + | + |
| 5 | 2011 | Nasal swab | t843 | + | – | – | + | + |
| 6 | 2004 | Sputum | t843 | + | – | – | + | + |
| 7 | 2010 | Nasal swab | t843 | + | – | – | + | + |
| 8 | 2007 | Mouth swab | t843 | + | – | – | + | + |
| 9 | 2010 | Nasal swab | t843 | + | – | – | + | + |
| 10 | 2011 | Nasal swab | t843 | + | – | – | + | + |
| 11 | 2011 | Joint aspirate | t843 | + | – | – | + | + |
| 12 | 2007 | Nasal swab | t978 | + | – | – | + | + |
| 13 | 2010 | Nasal swab | t7189 | + | – | – | + | + |
| 14 | 2009 | Nasal swab | t1773 | + | ND | – | + | + |
| Sheep | | | | | | – | | |
| 15 | 2010 | Unknown | t1535 | + | – | – | + | + |
| 16 | 2010 | Unknown | t1535 | + | – | – | + | + |

**spa*, *Staphylococcus aureus* protein A; MRSA, methicillin-resistant *S. aureus*; PBP2a, penicillin-binding protein 2a; SCC, staphylococcal cassette chromosome; +, positive; –, negative; ND, not done.
†ChromID MRSA-Plates (bioMérieux, Marcy-l'Étoile, France).

(cephalothin, n = 15; cefuroxime, n = 10; ceftriaxone, n = 2; cefepime, n = 9) to those classified as resistant.

We observed relatively low oxacillin/cefoxitin MICs for some of the *mecA*$_{LAG251}$-positive isolates (MIC 3 μg/mL, n = 1; MIC 4 μg/mL, n = 1; MIC 8 μg/mL, n = 3) compared with the MRSA reference strain ATCC 43300 (MIC 32 μg/mL). All isolates tested were susceptible to all non–β-lactam antibacterial agents, comprising glycopeptides, lipopeptides, fluoroquinolones, macrolides, lincosamides, oxazolidinones, rifampins, streptogramins, glycylcyclines, folate pathway inhibitors, aminoglycosides, and fosfomycin.

Until *mecA*$_{LGA251}$ is included as a diagnostic target in molecular MRSA detection tests, oxacillin/cefoxitin-resistant isolates determined to be methicillin-susceptible by traditional, culture-based susceptibility testing methods should not be disregarded, even if *mecA* and/or PBP2a tests fail to detect their targets. Susceptibility patterns of *mecA*$_{LGA251}$-positive *S. aureus* isolates revealed low MICs of oxacillin compared with those for MRSA of the classical *mecA* type. We presume this indicates an altered affinity of β-lactam antibacterial agents to the putative *mecA*$_{LGA251}$ gene product or a divergent expression of the gene. The choice and the dosage of antibacterial agents applicable for *S. aureus* infections should be reconsidered in light of this novel *mecA* homolog in molecular screening and identification tests. Studies are warranted to investigate the prevalence of this novel MRSA entity in and outside of hospitals in the human population and in livestock, its clinical effects, and its response to antibacterial agent therapy.

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# ESBL-Positive Enterobacteria Isolates in Drinking Water

**To the Editor:** Extended-spectrum β-lactamase (ESBL)–producing members of the family *Enterobacteriaceae* (enterobacteria) are a worldwide problem (*1*), but little data are available from central Africa (*2*). In recent years, ESBL-producing *Enterobacteriaceae* isolates have shifted from the hospital to the community and the environment (*1*). The aim of this study was to assess the presence of ESBL-producing *Enterobacteriaceae* isolates in sachet-packaged water bags sold as drinking water in the streets of Kinshasa, the capital of Democratic Republic of the Congo.

In November 2009 and June 2010, a total of 101 sachet-packaged water bags were bought from street vendors in 9 of 24 municipalities (covering residential areas and slums) of the city of Kinshasa. The bags were transported in ice coolers and processed within 4 hours of collection. We filtered 100 mL of each sample through 0.45-µm pore size filters (Sartorius, Goettingen, Germany). The filters were then transferred to an agar plate containing mEndoLES agar (Difco, Franklin Lakes, NJ, USA) and incubated at 35°C for 24 hours.

Growing colonies were subcultured on Kligler iron agar (Oxoid, Cambridge, UK), and gram-negative glucose-fermenting isolates were identified to the species level and assessed for antimicrobial drug susceptibility with Microscan NBC42 panels (Siemens Healthcare Diagnostics Inc., West Sacramento, CA, USA). Isolates labeled by Microscan as ESBL producers were confirmed by the double-disk method, which compared 1 disk containing cefotaxime with 1 disk containing cefotaxime and clavulanic acid and 1 disk containing ceftazidime with 1 disk containing ceftazidime and clavulanic acid (Rosco Diagnostica, Taastrup, Denmark), according to the Clinical and Laboratory Standards Institute guidelines (*3*). We used *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 as control strains. Detection and identification of ESBL-producing *bla* genes were carried out by using a commercial multiplex ligation PCR microarray CT 101 (Check-Points Health BV, Wageningen, the Netherlands) (*4*).

A total of 101 sachet-packaged water bags were purchased at random from different vendors: 88 sealed and branded bags (of 68 different brands) and 13 hand-tied unbranded bags. The precise origin of the water could not be determined because vendors are unlicensed resellers and most producers are not registered. Water bags lacked essential information such as contact addresses, batch number, and production and expiration dates. Because we also observed empty branded sheets of bags for sale on the market, we assumed that many of the branded bags are simply filled with tap water or water from other supplies without any prior treatment. None of the water bags tested for chlorine (36 of 101) contained free chlorine levels >0.1 mg/L. Nearly one third of the water bags were contaminated with *Enterobacteriaceae* isolates (22/88 branded bags and 9/13 hand-tied bags). The bags were obtained in townships and residential quarters.

Overall, 150 nonduplicate *Enterobacteriaceae* isolates were recovered. The main species were *K. pneumoniae* (56.0% of isolates found in 23/101 of water bags) and *Enterobacter* spp. (30.6%, in 20/101 water bags); *Citrobacter* spp. accounted for 4.7% of isolates and *E. coli* for 3.3%. Eight isolates (5.3 %) were confirmed as ESBL producers by antimicrobial drug susceptibility tests, and they were recovered from 2 branded and 2 hand-tied bags. The species, microarray results, and the associated drug resistance are listed in the Table. Five isolates carried $bla_{CTX-M}$ genes belonging to CTX-M1 group, and 3 isolates carried $bla_{SHV}$ variants. No TEM-ESBL genes were detected. On the basis of checkpoint results and previously validated data, we further categorized the SHV G238S mutation as SHV-2-like and the double SHV G238A + SHV E240K mutation as SHV-18 (*5*).

ESBL-producing *Enterobacteriaceae* isolates constitute a major public

Table. ESBL-producing *Enterobacteriaceae* recovered from sachet-packaged water bags in Kinshasa, Democratic Republic of the Congo*

| Isolate no. | Species | Microarray CT101 result | Associated resistance† | | Folate PI |
|---|---|---|---|---|---|
| | | | Aminoglycosides | Fluoroquinolones | |
| 44 | *Citrobacter freundii* | CTX-M1 group | AMK, GEN, TOB | CIP, LEV, MXF, NXN | T/S |
| 48 | *Citrobacter freundii* | CTX-M1 group | AMK, GEN, TOB | CIP, LEV, MXF, NXN | T/S |
| 152 | *Enterobacter cloacae* | SHV G238S (SHV-2 like) | NA | NA | T/S |
| 154 | *Klebsiella pneumoniae* | CTX-M1 group | GEN, TOB | CIP, MXF | T/S |
| 163 | *Citrobacter freundii* | CTX-M1 group | NA | MXF | T/S |
| 165 | *Klebsiella pneumoniae* | CTX-M1 group | GEN, TOB | NA | T/S |
| 170 | *Klebsiella pneumoniae* | SHV G238A+E240K(SHV-18) | GEN, TOB | MXF | NA |
| 171 | *Escherichia coli* | SHV G238A+E240K(SHV-18) | GEN, TOB | NA | NA |

*ESBL, extended-spectrum β-lactamase; PI, pathway inhibitors; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin; LEV, levofloxacin; MXF, moxifloxacin; NXN, norfloxacin; T/S, trimethoprim/sulfamethoxazole; NA, not applicable.
†Based on interpretive breakpoints as indicated in Clinical and Laboratory Standards Institute guidelines M100-S18, published January 2008.

health concern in industrialized and resource-poor settings. Few reports are available from Africa, although hospital-associated ESBL producers have been described in Cameroon and the Central African Republic (*6,7*). ESBL-producing bacteria have been recovered from different sources in the community, including food and companion animals (*8,9*), and 1 recent study from India reported that a substantial number of tap water samples were contaminated with carbapenemase $bla_{NDM-1}$ producing organisms (*10*).

Kinshasa is the second-largest city in sub-Saharan Africa. In 2008, of its estimated 8.7 million inhabitants, only 46%had access to safe drinking water, and 23% had access to improved sanitation facilities according to the World Bank. Opportunistic pathogens in drinking water and poor sanitary conditions may increase the risk of developing infectious enterocolitis for consumers, especially for those who are immunocompromised. It can eventually lead to chronic intestinal carriage of multidrug-resistant organisms. The presence of ESBL producers in the intestinal flora could also lead to horizontal transfer of drug resistance genes from commensal flora to enteric pathogens. This emergence of ESBL-producing bacteria and further community-associated infections poses a public threat, especially in low-resource countries where surveillance is suboptimal and empiric treatment of invasive infections often includes third-generation cephalosporins.

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# Novel *Chlamydiaceae* Disease in Captive Salamanders

**To the Editor:** Although 2 major diseases of amphibians, chytridiomycosis and ranavirosis, have been relatively well studied, enigmatic amphibian disease and death not attributable to any of the known amphibian diseases frequently occur (*1*). We describe an apparently new disease in salamanders that is associated with a novel genus within the family *Chlamydiaceae*.

The salamanders seen in our clinic belonged to 1 of the following species: *Salamandra corsica*, the Corsican fire salamander (5 animals from 1 collection); *Neurergus crocatus*, the yellow spotted newt (11 animals from 3 collections); or *N. strauchii*, Strauch's

spotted newt (6 animals from 2 collections). All salamanders were captive bred; housed in breeding colonies in private collections in Elsloo and Eindhoven, the Netherlands, Munich, Germany, and Brugge, Belgium; and 1–3 years of age.

Disease was characterized by anorexia, lethargy, edema, and markedly abnormal gait. Mortality rate was 100%. Animals in these collections had no histories of disease. All animals were in good nutritional condition. Necropsy did not yield any macroscopic lesions. All animals had mild intestinal nematode or protozoan infections. Results of real-time PCRs for iridoviruses in liver and skin (*2*) or *Batrachochytrium dendrobatidis* fungus of skin (*3*) were negative for all animals.

We placed liver suspensions from the dead salamanders on Columbia agar with 5% sheep blood and tryptic soy agar and then incubated the samples up to 14 days at 20°C. No consistent bacterial growth was observed. Histologic examination of 2 Corsican fire salamanders and 1 yellow spotted newt revealed hepatitis in 1 of the Corsican fire salamanders and the yellow spotted newt. Hepatitis was characterized by high numbers of melanomacrophages and a marked infiltration of granulocytic leukocytes. Immunohistochemical staining for chlamydia (IMAGEN Chlamydia; Oxoid, Basingstone, UK) showed cell-associated fluorescently stained aggregates in liver tissue, suggestive of Chlamydiales bacteria. Transmission electron microscopic examination of the liver of a yellow spotted newt revealed intracellular inclusions containing particles matching the morphology of reticulate or elementary bodies of *Chlamydiaceae* (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-1137-Techapp.pdf).

A PCR (*4*) to detect the 16S rRNA of all Chlamydiales bacteria, performed on liver tissue samples from all animals, yielded positive results in all 5 Corsican fire salamanders; in 4/7, 1/3, and 1/1 yellow spotted newts; and in 4/5 and 1/1 Strauch's spotted newts. For taxon identification, the 16S rRNA gene of the Chlamydiales bacteria was amplified and sequenced from the livers from 2 yellow spotted newts (1 from the collection in Elsloo, the Netherlands and 1 from the collection in Munich, Germany), 1 Strauch's spotted newt, and 5 Corsican fire salamanders.

The sequences shared >90% nt identity with the 16S rRNA gene of *C. abortus* B577 (GenBank accession no. D85709) and therefore can be identified as a member of the family *Chlamydiaceae* (*5*). The closest 16S rRNA similarity (92%) was observed with *C. psittaci* strain CPX0308 (AB285329). The sequence obtained from all spotted newt species specimens was identical (GenBank accession no. JN392920) but differed slightly (1%) from that obtained from the fire salamander species specimens (GenBank accession no. JN392919). These sequence differences point to the existence of multiple strains with possible host adaptation.

We determined the phylogenetic position of the novel taxon, named *Candidatus* Amphibiichlamydia salamandrae (online Technical Appendix), identified by using neighbor-joining analysis with Kodon software (Applied Maths, Sint-Martens-Latem, Belgium). The novel Chlamydiales forms a distinct branch in the well-supported monophyletic clade with the genera *Chlamydia* and *Candidatus* Clavochlamydia salmonicola (family *Chlamydiaceae*) (Figure). Maximum parsimony and unweighted pair group with arithmetic mean analyses yielded cladograms with the same topology (results not shown). Previous reports of members of the family *Chlamydiaceae* in amphibians concerned species occurring in other vertebrate taxa as well:

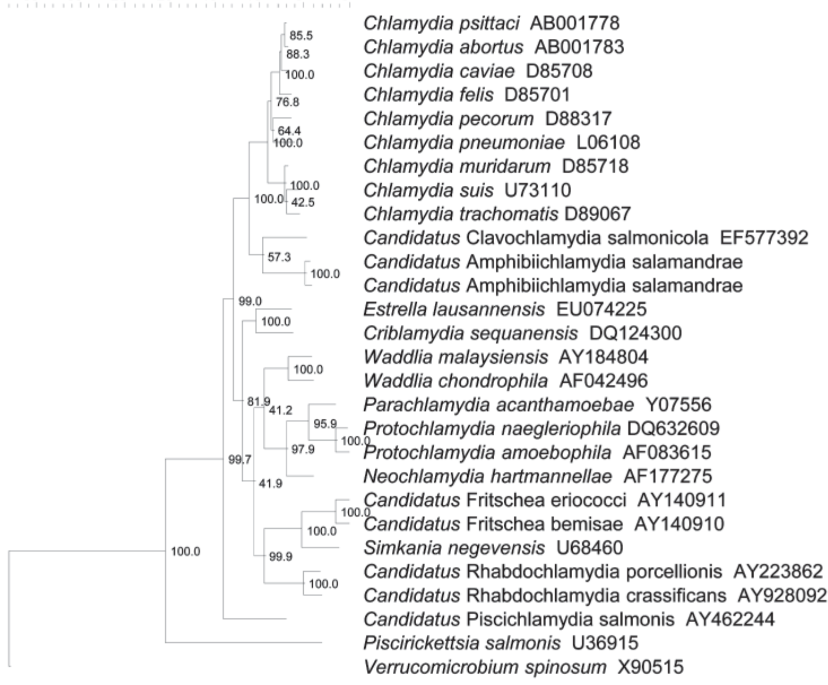


Figure. Topology of the novel amphibian *Chlamydiaceae* (*Candidatus* Amphibiichlamydia salamandrae) within the phylogenetic tree obtained by neighbor-joining and based on 16S rRNA gene data from representative species. Numbers show the percentage of times each branch was found in 1,000 bootstrap replicates. The tree has been rooted with *Verrucomicrobium spinosum* as outgroup. Scale bar indicates nucleotide substitutions per site.

*C. psittaci*, *C. pneumoniae, C. abortus*, and *C. suis* (*6–10*). To our knowledge, this member of the family *Chlamydiaceae* has been seen in amphibians, but not in other vertebrate hosts. The 16S rRNA analysis showed this taxon to belong to a clade with *Candidatus* Clavochlamydia salmonicola, a taxon found in fish. The phylogenetic position of the novel taxon in the family *Chlamydiaceae* thus roughly reflects the phylogenetic relation between the host species, providing evidence for host–bacterium co-evolution in the family *Chlamydiaceae*.

Although the results obtained are not conclusive with regard to the pathogenic potential of this novel genus and species of Chlamydiales, we were not able to attribute the clinical signs to any known disease. We therefore suggest that we discovered a novel bacterial taxon with possible considerable impact on amphibian health.

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# Novel Variant of Beilong Paramyxovirus in Rats, China

**To the Editor:** In 2003, two cDNA strands were identified in a human mesangial cell line during experimental screening for genes upregulated by angiotensin II (*1*). Sequence analysis showed that the strands were homologous to the matrix, fusion, and phosphoprotein genes of paramyxoviruses, suggesting the possibility of a novel paramyxovirus (*2,3*). Subsequent research found that these sequences, believed to originate from human kidney mesangial cell lines, were not amplifiable from such cell lines or human kidney samples but were amplifiable from a rat kidney mesangial cell line (*4*). Isolation and complete genome sequencing of the virus confirmed that it was a novel paramyxovirus of the subfamily *Paramyxovirinae*, named Beilong virus (BeV).

BeV is most closely related to J virus, discovered in autoculture of kidney tissue from a moribund house mouse, and Tailam virus from Sikkim rats (*5,6*). Because J virus and Tailam virus were found to originate in rodents and BeV was amplifiable from a rat kidney mesangial cell line, we hypothesized that BeV was a novel paramyxovirus originating in rats. To test this hypothesis, we conducted a territorywide molecular epidemiologic study of rats and other mammals to evaluate this novel paramyxovirus.

We tested 4,130 samples from 1,398 animals collected from various locations in Hong Kong, People's Republic of China, during September 2008–August 2009 (Table). These included 480 kidney, spleen, respiratory swab, and anal swab samples from 120 asymptomatic rats (105 brown rats [*Rattus norvegicus*] and 15 black rats [*R. rattus*]). To

prevent cross contamination, we used disposable scalpels, decontaminated the work surface, and used sterile gloves for each tissue sample. We performed RNA extraction and reverse transcription PCR by using strategies we previously published for discovery and epidemiologic study of paramyxoviruses (*6*–*9*).

We performed BeV screening by PCR amplification of a 440-bp fragment of the large (L) gene, located at the 5′ end of the genome and used specific primers (LPW9739 GGAGGATTCCCTCATAGAG AA-3′ and LPW9741 5′-CTCATAT GTATTTACATTTAAACCA-3′). The PCR mixture (25 μL) contained cDNA, PCR buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 3 mmol/L $MgCl_2$, and 0.01% gelatin), 200 μM of each dNTP, and 1.0 units of *Taq* polymerase (Applied Biosystems, Foster City, CA, USA). The mixtures were amplified in 60 cycles at 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min in an automated thermal cycler (Applied Biosystems).

BeV in the positive samples was confirmed by amplifying a 318-bp fragment of the nucleocapsid (N) gene of BeV, located at the 5′ end of the genome; by using specific primers (LPW10723 5′-TATATGGTTGAGAT YCTNATHGA-3′ and LPW10408 5′-CCATKGCRTAGCTCCADAG-3′) and experimental conditions described above. We confirmed the specificities of the primers by testing samples positive for Tailam virus (*6*), which all showed negative results.

Results of reverse transcription PCR for a 440-bp fragment in the large gene of BeV were positive for 40 kidney and 9 spleen samples from 43 rats (40 brown rats and 3 black rats). Sequencing and phylogenetic analysis showed 6–13 base differences between the sequences and the corresponding region in the large gene of BeV (GenBank accession no. NC_007803), suggesting that this is a novel variant of BeV in our locality.

Results of reverse transcription PCR for a 318-bp fragment in the N gene of BeV were positive in the same 40 kidney and 9 spleen samples from the 43 rats. Sequencing and phylogenetic analysis showed 1–9 base differences between the sequences and the corresponding region in the N gene of BeV. The kidney and spleen samples were positive in 4 brown rats and 2 black rats. The L and N gene sequences amplified from the kidney and spleen samples were identical in 5 of the 6 rats. However, in 1 brown rat, L and N gene sequences from the kidney and spleen samples differed by 4 and 6 bases, respectively, suggesting the possibility of 2 strains of BeV in the same rat. None of the samples from the other mammals were positive. The authenticity of the results was supported by identical results from 2 independent genes of the BeV genome, sequence variations in the L and N genes from the positive samples, and negative results from all other mammals tested.

This study suggests that BeV and its variants are endemic in brown rats and black rats, but it is not known whether transmission is vertical or horizontal. Detection of BeV and Tailam virus in kidney and spleen samples, but not respiratory or anal swabs, suggested that they are probably systemic viruses excreted in urine. Phylogenetic and genomic evidence support the grouping of BeV, Tailam virus, and J virus into a new genus of *Paramyxovirinae*. Distinctly, the genomes of all 3 viruses contain 8 genes (3′ -N-P/V/C-M-F-SH-TM-G-L-5′). We speculate that the ancestor of these closely related paramyxoviruses infected the common ancestor of rats and mice, with subsequent co-evolution and divergence with the host.

Table. Mammals screened for Beilong virus, People's Republic of China, September 2008–August 2009

| Animal | Sample type* |
|---|---|
| Bats, n = 502 | Throat swab, rectal swab |
| Cats, n = 130 | Nasal swab, rectal swab, urine, blood |
| Cattle, n = 100 | Nasal swab, rectal swab, liver, buffy coat, plasma |
| Dogs, n = 149 | Nasal swab, rectal swab, urine, blood |
| Hamsters, n = 49 | Throat swab, intestinal swab, kidney |
| Pigs, n = 100 | Nasal swab, rectal swab, liver, blood |
| Wild urban rodents, n = 120 | Rectal swab, throat swab, rectal swab, kidney, spleen |
| Wild rural rodents, n = 248 | Throat swab, rectal swab |

*All sample types listed for each animal were collected except wild rodents: 237 throat swab and 246 rectal swab samples were collected from 248 wild rodents.

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# Pneumococcal Serotype–specific Unresponsiveness in Vaccinated Child with Cochlear Implant

**To the Editor**: Approximately 100,000 persons worldwide have received cochlear implants for hearing loss, and more children now receive them than ever (*1*). Such children have a >30-fold increased risk for pneumococcal meningitis than the background rate (*1*,*2*). During 2006–2010, children born in the United Kingdom were offered the 7-valent pneumococcal conjugate vaccine (PCV7) at 2, 4, and 13 months of age (*3*). Those at high risk for invasive pneumococcal disease (IPD) were additionally offered the 23-valent pneumococcal polysaccharide vaccine (PPV23) at 2–5 years (*3*). We describe a fully vaccinated child with a cochlear implant in whom recurrent pneumococcal meningitis developed, caused by a vaccine serotype (i.e., vaccine failure). The child continues to have nonprotective antibody concentrations against the infecting serotype, despite further pneumococcal vaccination.

A previously healthy, appropriately vaccinated 23-month-old girl (Table) had a cochlear device implanted in the right ear after receiving (through the universal newborn hearing screening program) a diagnosis of profound, bilateral, sensorineural deafness. Two weeks later, she exhibited fever, lethargy, and drowsiness. On hospital admission, she had a peripheral blood leukocyte count of $19.3 \times 10^9$ cells/L, a neutrophil count of $17.0 \times 10^9$ cells/L, and C-reactive protein level 75 mg/L. Meningitis was diagnosed, and she received intravenous ceftriaxone but was too ill for a lumbar puncture. Blood cultures subsequently grew fully sensitive *Streptococcus pneumoniae*, later confirmed as serotype 4 by the national reference laboratory. She was discharged after 14 days of receiving intravenous antimicrobial drugs without complications.

At 24 months, she received a fourth dose of PCV7. Blood tests 1 month later showed good antibody responses to 6 PCV7 serotypes but not to serotype 4, which did not reach the putative protective level of $\geq$0.35 µg/mL antibody threshold (Table). At 28 months, she received 1 dose of PPV23 per national guidelines (*3*). Four months later, she was brought to the hospital with fever, rigors, drowsiness, and vomiting. Blood tests showed a leukocyte count of $24.4 \times 10^9$ cells/L, neutrophil count of $21.6 \times 10^9$ cells/L, and C-reactive protein level of 272 mg/L. Lumbar puncture performed the next day showed 890 leukocytes/mL (predominantly polymorphs), cerebrospinal fluid glucose level <1.1 mmol/L, protein level of 1.0 g/L, gram-positive diplococci on Gram staining, and positive PCR results for pneumococci, although cerebrospinal fluid culture was negative.

A blood culture grew fully sensitive *S. pneumoniae*, also confirmed by the national reference laboratory as serotype 4. She recovered after receiving intravenous ceftriaxone and oral rifampin for 2 weeks, followed by 4 weeks of oral amoxicillin and rifampin. She then received prophylactic oral penicillin for maintenance. Subsequently, an abdominal ultrasound confirmed the presence of a spleen, and her immunoglobulin concentrations were in the normal range. At 35 months, she received another dose of PCV7, and a blood test 1 month later showed variable but high responses to 6 of the PCV7 serotypes and no response to serotype 4 (Table). Moreover, nasopharyngeal swab specimens, obtained when the patient was 39 months old and receiving penicillin prophylaxis, were positive for serotype 4.

We described 8 previously healthy children with serotype-specific immune unresponsiveness after IPD, although a second IPD episode did not develop in these children (*4*). This phenomenon may result from large pneumococcal polysaccharide loads that deplete the memory B-cell pool and cause immune paralysis (*4*,*5*). In immunogenicity studies, some infants (1%–3%) remain unresponsive to conjugate vaccines (*5*). In a randomized controlled trial of PPV23 in 50–85-year-old persons, 3 vaccinated persons with culture-confirmed IPD had adequate pre- and postvaccination antibody concentrations to all but the infecting serotype, suggesting that they were unresponsive to the infecting serotype before vaccination (*6*). In infants, recent randomized controlled trials have found that nasopharyngeal carriage at first dose of PCV7 resulted in significantly lower IgG responses to that specific serotype than occurred with noncarriers or carriers of other serotypes, possibly because of high carriage–induced polysaccharide loads (*7*,*8*). Moreover, unresponsiveness was only partially overcome by the 12-month PCV7 booster (*7*).

This case raises key questions regarding long-term clinical management of children with serotype-specific immune unresponsiveness after vaccination or infection. The case is further complicated by the patient's cochlear implant, which may have been the source of infection (*9*), as well as evidence of nasopharyngeal carriage while the patient was receiving antimicrobial drug prophylaxis and recurrence of meningitis caused by the same serotype. However, her ability to respond to the other 6 PCV7 serotypes, normal immunoglobulin concentrations, no previous history of recurrent infections, and presence of a spleen all provide evidence against an underlying immune problem.

Further pneumococcal vaccination of this patient is unlikely to reverse the unresponsiveness, which may persist for years (*4*,*5*). Studies to clarify the immune mechanisms underlying unresponsiveness and strategies to reverse this phenomenon are, therefore, urgently warranted. In the meantime, we recommend that the infecting pneumococcal serotype be determined in children with IPD and that, when possible, those infected with a vaccine-related strain (particularly children with risk factors) have serotype-specific pneumococcal antibodies measured after infection. Appropriate measures to prevent recurrent IPD should also be taken, such as removal of potentially infected devices or long-term prophylaxis with antimicrobial drugs.

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Table. Pneumococcal serotype-specific IgG concentrations in 2-year-old child with recurrent pneumococcal meningitis, United Kingdom*

| Patient age, mo | Event | Serotype-specific IgG, µg/mL | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 4 | 6B | 9V | 14 | 18C | 19F | 23F |
| 1.9 | PCV7 dose 1 | | | | | | | |
| 3.9 | PCV7 dose 2 | | | | | | | |
| 13.4 | PCV7 dose 3 | | | | | | | |
| 22.8 | Cochlear implant | | | | | | | |
| 23.4 | Pneumococcal meningitis (episode 1) | | | | | | | |
| 24.4 | PCV7 dose 4 | | | | | | | |
| 25.6 | Pneumococcal serologic testing | 0.12 | 27.6 | 23.0 | 36.9 | 13.8 | 57.0 | 41.1 |
| 27.8 | PPV23 dose 1 | | | | | | | |
| 28.8 | Pneumococcal serologic testing | 0.18 | 14.2 | 13.1 | 30.4 | 11.4 | 11.7 | 32.0 |
| 32.0 | Pneumococcal meningitis (episode 2) | | | | | | | |
| 35.1 | PCV7 dose 5 | | | | | | | |
| 36.1 | Pneumococcal serologic testing | 0.20 | 18.40 | 7.12 | 12.50 | 12.40 | 2.78 | 37.1 |
| 39.0 | PCR positive nasopharyngeal swab specimen for *Streptococcus pneumoniae* serotype 4 | | | | | | | |

*PCV7, 7-valent pneumococcal conjugate vaccine; PPV23, 23-valent pneumococcal polysaccharide vaccine. Blank spaces indicate not tested.

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# African Swine Fever Virus Strain Georgia 2007/1 in *Ornithodoros erraticus* Ticks

**To the Editor:** African swine fever virus (ASFV) causes a notifiable disease in domestic pigs for which no treatment or vaccine is available, resulting in a mortality rate of ≤100%. In 2007 ASFV was detected in the Caucasus region, first in Georgia and subsequently in Armenia, Azerbaijan, and many parts of Russia, including regions that border other countries in Europe and Asia (*1*).

Most field strains of ASFV can persistently infect *Ornithodoros* ticks, including the species *O. erraticus* in southern Europe (*2*), and ASFV has been isolated from ticks collected >5 years after the last confirmed case in an outbreak (*3*). These ticks can feed on alternative hosts, evade eradication attempts (such as acaricide application and flamethrowers), and survive for up to 15 years (*1*). Although *Ornithodoros* species have been reported in the Caucasus region, their distribution is not well known (*1*). It is also not known if the Georgia 2007/1 ASFV strain responsible for continuing outbreaks in the Caucasus region can replicate in ticks. Thus, we conducted a study to determine whether the Georgia 2007/1 isolate of ASFV can replicate in *Ornithodoros* ticks.

*O. erraticus* ticks from Alentejo, Portugal (provided by Fernando Boinas, Universidade Técnica de Lisboa in Lisbon, Portugal) were sorted into groups of 10 adults or fifth-instar nymphs, placed into 60-mL containers covered with nylon cloth (16-cm mesh), and maintained at 85% relative humidity and 27°C for 18 months without feeding. Heparinized pig blood containing antibacterial drugs and fungicide (10 µL of streptomycin [10,000 IU/mL], 10 µL of amphotericin B [250 µg/mL], and 5 µL of neomycin [10 mg/mL 0.9% NaCl]/mL of blood) was mixed with the Georgia 2007/1 isolate (*4*) or the OUR T88/1 isolate (*5*) as a positive control to obtain virus titers of 4 $\log_{10}$ or 6 $\log_{10}$ 50% hemadsorbing doses ($HAD_{50}$)/mL blood. These titers were within the observed range in naturally infected pigs (*6*), and thus simulated the field situation.

Ticks were fed infected blood by using a Hemotek membrane-feeding system (Discovery Workshops, Accrington, UK). Meal reservoirs were covered with stretched Parafilm that was wiped with a thin film of uninfected blood to encourage feeding. Pots of ticks were placed on the membrane and allowed to feed for 20 minutes.

Immediately after and 3, 6, 9, and 12 weeks after feeding, 10 ticks from each feeding group were killed by freezing in dry ice. After being washed with a detergent solution and phosphate-buffered saline, ticks were placed individually in tubes with 200 µL of RPMI medium (Sigma-Aldrich Company Ltd., Gillingham, UK), a 3 mm-diameter stainless steel ball (Dejay Distribution Ltd., Launceston, UK), and 1-mm silicon carbide particles (Stratech Scientific Ltd, Newmarket, UK). They were then homogenized by shaking for 5 cycles of 3 minutes at 25-Hz frequency using a TissueLyser (QIAGEN, Valencia, CA, USA). To complete a 1-mL volume, 800 µL of RPMI medium was added to the tubes after centrifuging 2× for 30 seconds at 2,000 rpm. Supernatants were transferred to fresh tubes and centrifuged for 5 minutes at 1,000 × *g*.

Virus titers were estimated on porcine bone marrow cells (*7*) and expressed as $\log_{10}$ $HAD_{50}$ per tick. Previous studies suggest that it takes 3–4 weeks for ticks to completely digest and clear ingested blood and that virus isolated after this period is due to viral replication (*5,6*). A general

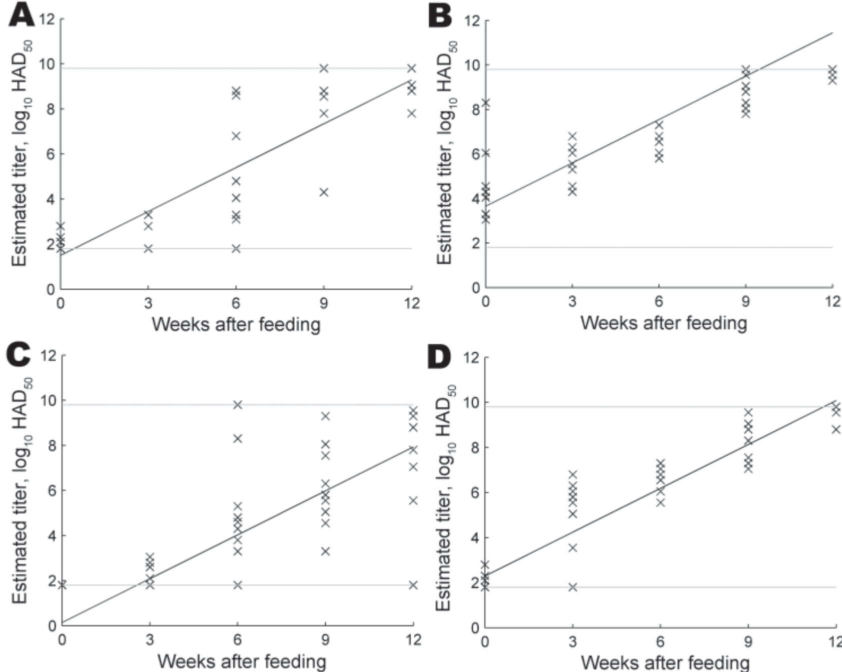


Figure. Predicted regression for each isolate–dose combination is shown. A) Ticks fed on African swine fever virus (ASFV) strain OUR T88/1 at 4 $log_{10}$ 50% hemadsorbing doses ($HAD_{50}$)/mL. B) Ticks fed on ASFV strain OUR T88/1 at 6 $log_{10}$ $HAD_{50}$/mL. C) Ticks fed on ASFV strain Georgia 2007/1 at 4 $log_{10}$ $HAD_{50}$/mL. D) Ticks fed on ASFV strain Georgia 2007/1 at 6 $log_{10}$ $HAD_{50}$/mL. Crosses indicate experimental results, and solid line indicates model prediction. Dashed horizontal lines show the limits of the tissue culture sensitivity (lower limit 1.8 $log_{10}$ $HAD_{50}$ and upper limit 9.8 $log_{10}$ $HAD_{50}$).

linear model, fitted via maximum likelihood, was used to assess the effects of isolate, dose, time after feeding, and interaction between isolate and time after feeding on the viral titer in the tick. Confidence intervals were calculated by profile likelihood.

Results showed that the Georgia 2007/1 strain can replicate in the *O. erraticus* tick. We recovered virus titers of <1.8 to >9.8 $log_{10}$ $HAD_{50}$ per tick. Ticks that fed on blood containing 6 $log_{10}$ $HAD_{50}$ ASFV on average had virus titers 2.15 $log_{10}$ $HAD_{50}$ higher than those for ticks that fed on blood containing 4 $log_{10}$ $HAD_{50}$/mL. Over time, the average titer for both isolates increased at an estimated rate of 0.65 $log_{10}$ $HAD_{50}$/week, indicating replication. Statistical analysis suggested that immediately after feeding, ticks fed on the Georgia 2007/1 strain contained 1.36 $log_{10}$ $HAD_{50}$ less virus than those fed on the OUR T88/1 isolate, but we detected no statistically significant difference in the replication rates of the 2 isolates. Parameter estimates are shown in the Table and the model fit is shown in the Figure.

Table. General linear model of the effects of different parameters on the titer of ASFV in experimentally infected *Ornithodoros erraticus* ticks*

| Parameter | Maximum likelihood estimator (95% CI) |
|---|---|
| Constant | 1.4985 (0.7084 to 2.2610)† |
| ASFV strain | –1.3620 (–2.4007 to –0.3482)† |
| Dose | 2.1538 (1.5889 to 2.7316)† |
| Time after feeding (effect per week) | 0.6494 (0.5546 to 0.7481)† |
| Isolate–time interaction | –0.0025 (–0.1400 to 0.1363)‡ |

*Ticks were fed pig blood with 4 $log_{10}$ or 6 $log_{10}$ $HAD_{50}$/mL ASFV strain Georgia 2007/1 or strain OURT88/1. ASFV, African swine fever virus; $HAD_{50}$, 50% hemadsorbing doses.
†Statistically significant, $p<0.01$.
‡Not significant ($p>0.05$).

The whole-tick titers reported in this study are consistent with those from previous studies (*5,6*). However, ≥9 weeks after the ticks fed, we observed higher titers than those reported (*5*), and many results showed ≥9.8 $log_{10}$ $HAD_{50}$ per tick. The TissueLyser may have been more effective at releasing ASFV from tick tissues than previously used methods. Our results suggest that virus replication within the ticks began by 3 weeks after feeding on infected blood; this timing is consistent with that in previous studies (*5,8,9*).

We observed high viral titers for ≥12 weeks after infection. Previous studies showed that ASFV can persist at high titers for 20 weeks after infection (*10*). We demonstrated that ASFV Georgia 2007/1 isolate can replicate efficiently in ticks. This finding highlights the importance of clarifying the distribution of *Ornithodoros* species ticks in the Russian Federation and Caucasus region and the relationship of these ticks to species susceptible to ASFV.

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# Apparent Triclabendazole-Resistant Human *Fasciola hepatica* Infection, the Netherlands

**To the Editor:** In December 2007, a 71-year-old sheep farmer sought care with a 4-month history of intermittent right upper quadrant pain, night sweats, anorexia, and a 5-kg weight loss. His medical history was unremarkable, and he had not traveled outside the Netherlands for ≈30 years. Physical examination revealed no abnormalities.

Blood tests showed an elevated erythrocyte sedimentation rate of 35 mm/h (reference 1–15 mm/h), normocytic anemia (hemoglobin 7.0 mmol/L [reference 8.5–11 mmol/L]), and eosinophilia ($2.5 \times 10^9$ cells/L [reference $0.0–0.5 \times 10^9$ cells/L]). Levels of alkaline phosphatase, γ-glutamyl transferase, and alanine aminotransferase were elevated (146 U/L [reference 10–120 U/L], 143 U/L [reference 5–50 IU/L], and 54 U/L [reference 0–45 U/L], respectively). Levels of bilirubin and aspartate aminotransferase were normal. Computed tomography of the liver showed several irregularly shaped low-attenuating lesions ranging in size from 1 to 4 cm. High titers of IgG (640 [cutoff 40], determined by enzyme immunoassay) against *Fasciola hepatica* were detected. Subsequently, *F. hepatica* eggs were detected in fecal samples.

The patient, who spontaneously had become asymptomatic shortly after seeking care, was treated unsuccessfully with the benzimidazole derivative triclabendazole (TCBZ) on 3 separate occasions during the next 2 years. He was first treated with a single dose of 10 mg/kg TCBZ (Fasinex suspension; Novartis Animal Health Ltd., Surrey, UK), then with 2 doses 24 hours apart, and on the last occasion with 2 doses of TCBZ (Egaten; Sipharm Sisseln AG, Sisseln, Switzerland) 10 mg/kg 12 hours apart; the last 4 treatments were taken with food. Feces remained positive for *F. hepatica* eggs after each treatment. IgG titers remained positive (320, by enzyme immunoassay), and flukes could be visualized by ultrasound in the gallbladder and common bile duct (Figure). Thereafter, the patient was treated with nitazoxanide (500 mg 2×/d for 7 days); however, fecal samples remained positive for *F. hepatica* eggs. Lastly, after recent experiments of a combination therapy in a rat model (*1*), we treated the patient with TCBZ (Egaten, 10 mg/kg) combined with ketoconazol 10 mg/kg taken with food. Still, his feces remained positive for *F. hepatica* eggs.

Fascioliasis is a zoonotic disease caused by the foodborne trematode *F. hepatica* or *F. gigantica*, which has a complex life cycle and mainly affects sheep and cattle (*2*). Eggs of the adult worms (2–4 cm) that live in the bile ducts of the final host are excreted in the feces and develop into larvae (miracidia) in water. The miracidia then penetrate, and further develop in, snails of the family Lymnaeidae. Free-swimming cercariae exit the snail and attach to aquatic vegetation, where they encyst as metacercariae. After

ingestion by the host, they excyst in the intestine and migrate through the intestinal wall to the liver, where they mature into adult flatworms that reside in the bile ducts (*2*).

Fascioliasis affects millions of humans worldwide (*3*); however, fascioliasis acquired in the Netherlands has been reported only sporadically (*4*), even though *F. hepatica* infection in sheep and cattle is prevalent there (*5*). The patient in this report had not eaten watercress or other aquatic plants and had not ingested ditchwater. However, he had worked in and around ditches on farms in the area, admitted chewing grass sporadically, and might have occasionally ingested vegetables previously fertilized with livestock manure. The patient remains asymptomatic but infected.

TCBZ is the treatment of choice for fascioliasis. In a review by Keiser et al. (*6*), the efficacy of treatment with TCBZ was shown to yield egg clearance in 78%–100% of patients after 1 dose of 10 mg/kg and in 92%–100% after 2 doses of 10 mg/kg each 12 or 24 hours apart. In livestock, TCBZ resistance is being reported increasingly (*7*). Mass treatment of sheep and cattle with TCBZ (Fasinex) or in combination with other anthelmintic drugs is common in the Netherlands (L. Moll, pers. comm.), and the first cases of resistance were described in 1998 in sheep and cattle in the province of North Holland, the area of residence of the patient reported in this study (*8*,*9*). During 1998–2004, resistance to TCBZ, proven by fecal egg count reduction tests, was found on 14 farms in the same area (*5*).

The findings in this case are most likely explained by TCBZ resistance, although we note that repeated TCBZ courses are not 100% effective against fascioliasis (*6*). Re-infection can be excluded because fecal samples were tested for eggs 1–3 months after each treatment. This description of apparent TCBZ-resistant fascioliasis in a human highlights the human health implications of (massive) anthelmintic use in livestock.

Further studies on TCBZ resistance and on therapeutics for fascioliasis need to be conducted. In addition, the role of antimicrobial drugs in the treatment of livestock needs to be more rigorously evaluated.

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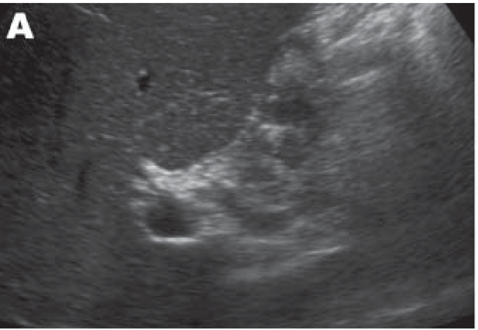


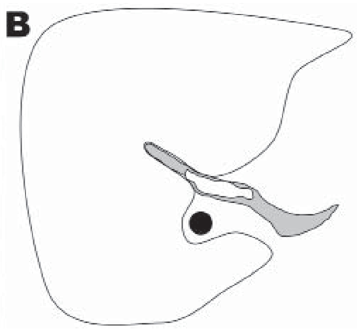


Figure. A) Ultrasound of the liver of a patient with *Fasciola hepatica* infection, the Netherlands. B) Drawing of A; depicted are the liver (white), the common bile duct (gray), and the portal vein (black). A fluke (white), measuring 2.5–3 cm long, is identified in the common bile duct.

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# Possibility of Leishmaniasis Transmission in Jura, France

**To the Editor:** The report of a human cutaneous leishmaniasis case acquired in Clairvaux-les-lacs (*1*) led us to carry out an investigation with the veterinary clinics in Jura Department, France. Clairvaux-les-lacs is a lakeside resort located in Jura, one of the areas in France with the coldest average temperatures, and is clearly located outside the usual leishmaniasis-endemic area. At least 31 cases of canine leishmaniasis were diagnosed by veterinary clinics in Jura during 2007–2011. Because these dogs were native of or traveled in the leishmaniasis-endemic area along the Mediterranean Sea, all veterinarians considered the infections as acquired outside Jura.

Although phlebotomine sand flies have not been reported in Jura to date, *Phlebotomus perniciosus* sand flies, proven vectors of leishmaniasis, have been found in 2 areas neighboring Jura: Côte-d'Or and Saône-et-Loire (*2*,*3*). We have also recently caught *P. mascittii* sand flies, a species with an unknown vectorial competence, in the Swiss region of Jura, Alsace, Champagne-Ardennes, and Belgium. Therefore, the presence of sand flies in Jura, particularly in wet and milder microclimatic areas (as Clairvaux-les-lacs), is likely, and canine infections could have been acquired locally.

A recent model predicted that new at-risk areas are mostly located in western France along the Atlantic coast (*4*). In accordance with this model, we report new foci of autochthonous canine leishmaniasis in Deux Sèvres, Loire-Atlantique, and Loiret. Canine leishmaniasis cases contracted in the Rhine Valley in Germany (*5*) and the canine cases in Jura argue for a northeastern spread of the disease-endemic area along the Rhone-Rhine axis and mild microclimatic niches. Entomologic and serologic surveys will be carried out in summer 2012 in Jura to look for evidence of possible indigenous transmission of leishmaniasis. These data should supplement the current model of northern spread of leishmaniasis-endemic areas.

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# Etymologia: Prion

**To the Editor:** The January 2012 Etymologia section might confuse readers because it incorrectly reports that "prion" describes a noninfectious agent (*1*). In fact, prion—pronounced *pree'-on*—is a term coined in 1982 by Nobel laureate Stanley Prusiner to describe the novel infectious agent responsible for scrapie, a transmissble neurodegenerative disorder of sheep and goats. He proposed his new term to underscore that the agents are "small ***pro***teinaceous ***in***fectious particles" resistant to procedures that attack nucleic acids (*2*). In his seminal article, he summarized experimental data indicating that the molecular properties of this infectious agent differed from those of other infectious agents, including viruses, viroids, and plasmids; he proposed the word prion to replace other terms then in circulation, such as "unconventional virus" or "unusual slow virus–like agent."

Although Dr. Prusiner acknowledged that he could not exclude the possibility of a small nucleic acid contained within the interior of the prion particle, now 3 decades later, no nucleic acid in the agent has yet been identified. Increasingly accepted in the scientific community, prions are now considered to be a class of misfolded proteinaceous, infectious agents responsible for several types of human and animal transmissible spongiform encephalopathies. Their evolving defining characteristics classically include at least partial protease resistance, insolubility, and transmissibility. The term, prions, usually refers to the complete transmissible proteinaceous particles in nature or to their classically present, transmissible, protease-resistant oligomer cores, composed of protein fragments with molecular masses of ≈27–30 kDa.

Adding confusion to the terminology, it has become customary

for prion researchers to refer to the normal nonpathogenic conformation of prions as "cellular prion proteins" (*3*). When these normal cellular prion precursors convert to pathogenic prion proteins, the transmissible conformations are characterized by β-pleated sheets rather than the normal α-helix structure, and they do not elicit an immune response (*4*).

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# Hepatitis E Virus Infection in Sheltered Homeless Persons, France

**To the Editor:** Kaba et al. (*1*) reported a seroprevalence of 11.6% for hepatitis E virus (HEV) among homeless persons in the city of Marseille, located in southern France, and a multivariate analysis suggested that injection drug use (IDU) was an independent risk factor for HEV transmission. We disagree with this reported finding.

We conducted a retrospective subanalysis of results from a multicenter therapeutic trial assessing HEV seroprevalence among HIV/hepatitis C co-infected patients in France (*2*). Serum samples from 84 IDU patients, enrolled during 2000–2002 were stored at −80°C. The mean ± SD age of the patients was 39 ± 4 years; 53 (63%) were men, 19 (23%) were born outside France, and 38 (45%) were living in southern France. HEV antibodies were tested with the same assay as that used by Kaba et al. (*1*), and HEV RNA was detected by using a real-time reverse transcription PCR amplifying open reading frame 3 (*3*). None of the patients had detectable IgM against HEV or HEV RNA. Test results for 3 (3.6%) patients were positive for HEV IgG. Two of them lived in southern France, resulting in a 5.3% (2/38) HEV prevalence for IDU patients living in this region, where HEV IgG prevalence for healthy blood donors has reportedly ranged from 9% to 16.6% (*4*).

The difference between our study, which demonstrated low HEV IgG prevalence in IDU patients, even in southern France, and the results from Kaba et al. (*1*) must be interpreted with caution because there were several epidemiologic differences between the 2 populations. Moreover, there is a risk for false-negative serologic results for HIV patients because of impaired immunity, and the predictive value of serologic testing is probably low because of the artificially low HEV prevalence reported for this population. Despite these limitations, our study suggests that the high prevalence of HEV infection among homeless persons in southern France was not influenced by IDU, but reflected the general epidemiology of HEV in this region.

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**In Response:** The letter of Larrat et al. (*1*) raises interesting questions, but we disagree with the authors' conclusion. Their study and ours investigated the seroprevalence of infection with hepatitis E virus (HEV) (*1*,*2*); however, the 2 studies examined different populations. We studied homeless persons, for whom analysis of risk factors associated with HEV infection emphasized injection drug use (*2*). This represented independent data: injection drug use was a behavior associated with increased anti-HEV prevalence; a causal relationship between injection drug use and hepatitis E was not inferred. In contrast to our study population, the population studied by Larrat et al. comprised patients who were co-infected with HIV and hepatitis C virus and who reported injection drug use as the route of HIV or hepatitis C virus transmission: a distinctly different population from homeless persons (*1*). It is likely that behavior of HIV-positive and HIV-negative intravenous drug users is not the same. Moreover, late seroconversion, persistent seronegativity, and seroreversion of IgG against HEV have been reported for severely immunocompromized patients, including some infected with HIV (*3*–*5*), which brings up the question as to whether prevalence of IgG against HEV is underestimated among severely immunocompromised persons infected with HIV.

Seroprevalence studies of different populations, especially those with differing immune responses, cannot lead except by chance to the same result. Of note, we recently reported that HEV seroprevalence was 2.3% among injection drug users infected with HIV (*5*).

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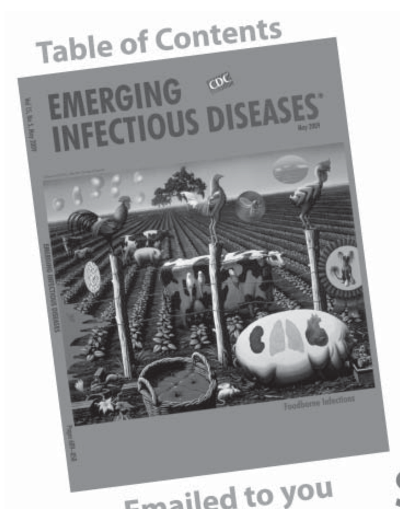


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# Mayo Clinic Infectious Diseases Board Review

**Zelalem Temesgen**

**Oxford University Press, New York, NY, USA, 2011**
**ISBN: 978-0-19-982762-6**
**Pages: 584; Price: US $99.99, UK £58.50**

This 600-page board review book provides an excellent synopsis of infectious diseases. It is divided into 4 sections, each with a questions and answers section: General, Etiologic Agents, Select Major Clinical Syndromes, and Special Hosts and Situations. There are almost 200 questions in the book; however, they are not written in the American Board of Internal Medicine test format. Compared with the bulky binders filled with copies of electronic presentations that I took home from a live board review course, the Mayo Clinic book is a more useful and convenient desk reference.

The infectious disease information is presented in a way that facilitates understanding, not just memorization. Although organized differently, the content of the book is comparable to that presented in the live course. Some chapters were exceptional (e.g., Infections of the Central Nervous System, Obstetric and Gynecologic Issues Related to Infectious Diseases). I showed the book to an infectious disease fellow who passed the boards in 2011 with a score of 99%; that bright young fellow noted that the coverage of infectious diseases in transplant recipients and in patients with hematologic malignancies is "sketchy."

In terms of practical examination preparation, the book is not as good as the live course when it comes to training test takers to recognize clues within the test. The book could more explicitly emphasize combinations of clues, or "buzz" words, that should trigger certain associations for the test taker (e.g., if question stem includes "cirrhosis and oysters," look for "*Vibrio vulnificus*" among the answer choices). In addition, the book does not cover non–infectious disease syndromes masquerading as infections (e.g., rheumatologic syndromes), which, along with ethics, comprise up to 10% of the examination. The chapter on liver infections left me wanting more help distinguishing various types of viral hepatitis, i.e., hepatitis A–E, from other infectious causes of hepatitis, such as leptospirosis, as well as non-infectious causes of hepatitis. The book seems to have omitted certain important points, such as when vaccine against hepatitis A virus is recommended as a postexposure intervention.

In conclusion, the Mayo Clinic Infectious Diseases Board Review book is an excellent means to solidifying one's infectious disease knowledge, and it could be a useful as an adjunct review tool. However, in terms of preparing for actual examination-style questions and for assuring that the full breadth of required information is covered, test takers will not want to rely on this book alone.

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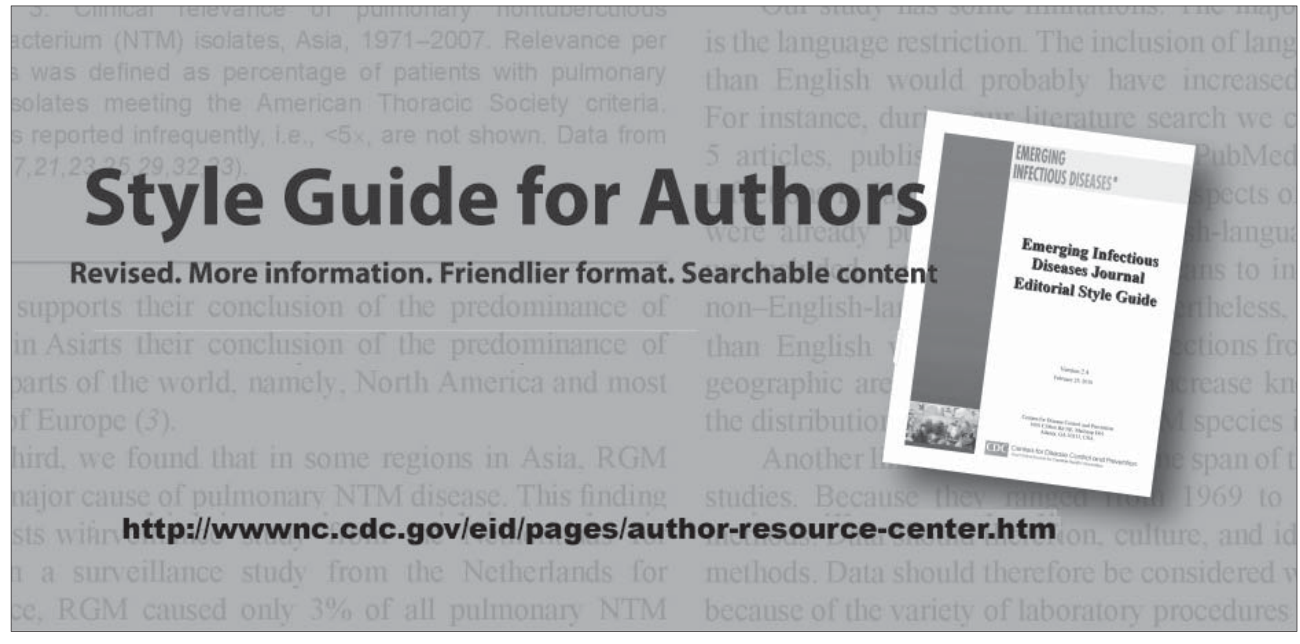

**Gerard van Kuijl, Dutch painter active in Rome (1604–1673) *Narcissus* (c. 1645) Oil on canvas (114 cm × 143 cm)** Collection of The John and Mable Ringling Museum of Art, http://www.ringling.org/ The State Art Museum of Florida, a division of Florida State University

# I rhyme / To see myself, to set the darkness echoing[1]

**Polyxeni Potter**

"As a child, they could not keep me from wells," wrote Seamus Heaney in his version of the Narcissus myth. "I loved the dark drop, the trapped sky, the smells / of waterweed, fungus and dank moss." In countless versions, the ancient myth strikes a universal chord: person sees self, meets death. Ovid told of handsome Narcissus and Echo, the nymph who fell in love when she saw him "chasing frightened deer into his nets." Rejected, she wasted away, until nothing was left but her voice, "heard by all." He, "Tired from both his enthusiasm for hunting and from the heat," rested a spell, caught glimpse of his reflection in a pool of water, and fell in love with "all the things for which he himself is admired." Unable to tear himself from the fateful reflection, he too wasted away. At this spot later sprouted narcissus, the flower.

Favored by poet and artist alike, the story intrigues anyone who searches for or reconstructs the self. In art, variations abound, among them one by the great Michelangelo Merisi da Caravaggio (1571–1610). Contrary to the conventions of his age, he painted directly from posed models, a practice that cultivated a new relationship between painting and viewer by promoting art not as fiction but as an extension of everyday experience, the physical content enriched with psychological tension. Gerard van Kuijl, Dutch painter and follower of Caravaggio, might have seen the master's *Narcissus* (1597) when he lived in Rome from 1629 to 1631.

In the 17th century, many artists from the northern Low Countries worked abroad or were influenced by others who had traveled and returned, bringing new styles to the local market. Their work strayed from the polders, woods, and dunes of the Dutch Golden Age to the biblical and secular, with human figures dominating the canvas. These artists exerted a lasting influence by introducing one of the main currents of baroque art into the Netherlands. The Caravaggists were mainly artists from Utrecht, who visited Italy and worked in the style of Caravaggio, characterized

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[1]From "Personal Helicon" by Seamus Heaney (www.ibiblio.org/ipa/poems/heaney/personal_helicon.php).

by realist drama and strong interplay of light and dark. This style also prevailed outside Utrecht, affecting Rembrandt and his followers.

In van Kuijl's *Narcissus*, expert shading betrays Caravaggist influence as do the baroque shapes and style. In both the Caravaggio and van Kuijl renditions, the figure is wrapped in a mystical, isolating, introspective dark. But, despite the striking similarities, van Kuijl's approach is individualized. While Caravaggio moved the figure into his own times showing no traces of classical attire, van Kuijl maintained topical decorum and a trace of the pastoral. Overall, it seems as if the two images represented a sequence. In the earlier painting the figure is actively engaged with his reflection, almost interactive, agile, embracing. In van Kuijl's work, having given into the overwhelming attraction, the figure is entranced, dreamy, stochastic.

The theme of Narcissus is not new to science, having been exhaustively addressed in psychoanalysis and come down to us as narcissism and the narcissistic personality. In one iteration, the theme overlaps with the ever-popular myth of Pygmalion, the sculptor in antiquity, who fell in love not with his image but with his work—a female statue he created, one so perfect that it was, in his estimation, more beautiful than any woman could ever be.

Breathing life in or animating a work of art was not the domain alone of Pygmalion. Lyric poet Pindar in his seventh Olympic Ode wrote, "The animated figures stand / Adorning every public street / And seem to breathe in stone, or move their marble / feet." Daedalus used to install voice in his statues, and Hephaestus created automata for his workshop. But these mechanical attempts with lifeless objects pale before more recent achievements, no less in modern medicine, which breathe life into dying human bodies with grafts, transfusions, and transplants, extending their tenure and the resilience of the species.

"Man," wrote Johann Wolfgang von Goethe in his novel Elective Affinities, "is a true Narcissus. He makes the whole world his mirror." The philosopher's interest was literary, an opportunity to unravel personal and social processes and interpret the meaning of human actions and events. He thought that, like the young Seamus Heaney, man could not pass up an opportunity "To stare, big-eyed Narcissus, into some spring." Nevertheless, as an adult, the poet himself found his early fascination with the well undignified. "I rhyme," he professed, "To see myself, to set the darkness echoing."

Goethe had no way of knowing that the time would come when humans would literally be able to recreate that which so fascinated them as myth and metaphor—not only recreate their image in a poem or, like Pygmalion, in art but also in the flesh. For loving one's creation is certainly easy to fall into, even in science. A more resilient human with healthier and longer lasting parts is the reflection we look for in the well, a reflection too of improved medical knowledge, expertise, and technology: properly used antimicrobial drugs; preventive screening; and growth hormone and dura mater grafts from cadavers, the bold modern equivalent of magic and automata in statues. Yet the echo we expect to hear from the darkness is often interrupted by emerging pathogens and often, unawares, by ourselves.

The history of medicine is filled with examples of unintended consequences. A concern since the time of Hippocrates, "to do no harm" is a continuing chapter with an abundance of contemporary examples. In this issue of the journal, reliance on a single class of antimicrobial drugs for treatment of some infections heightens our vulnerability to emergence of resistance, requiring more treatment options. Preoperatively acquired emerging pathogens complicate liver transplantation, a problem threatening to increase, despite adequate infection control measures. On the other hand, comprehensive current tallies of global incidence of iatrogenic Creutzfeldt-Jakob disease identified no new sources of disease, indicating that current practices should continue to minimize the risk until blood screening is validated for human use and suggesting that, despite setbacks that make that glimpse of perfect self fatal, science diligently applied can still set the darkness echoing.

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## Iatrogenic Creutzfeldt-Jakob Disease, Final Assessment

## CME Questions

**1. Your patient is a 50-year-old woman with a complicated medical history. She has required treatment with multiple biologic agents and had surgery with placement of a cadaveric ligament in her knee.**

**She read an article regarding prion disease and is concerned regarding her risk for illness. According to the current review, what have been the principal sources of iatrogenic Creutzfeldt-Jakob disease (CJD)?**

A. Corneal transplants and packed red blood cells
B. Instruments and gonadotropins
C. Dura mater grafts and growth hormone
D. Packed red blood cells and platelets

**2. Most cases of iatrogenic CJD caused by human growth hormone are reported in which of the following countries?**

A. Ghana, Ivory Coast, and Nigeria
B. Vietnam, Cambodia, and Laos
C. France, the United Kingdom, and the United States
D. Brazil, Colombia, and Peru

**3. What can you tell this patient about characteristics of iatrogenic CJD?**

A. Homozygotes for the methionine valine polymorphism had longer incubation times
B. The mean incubation period is approximately 2 years
C. Dementia was the most common early manifestation of CJD
D. One manufacturer accounted for nearly all infections associated with dura mater grafts

**4. What are the most prominent new threats for CJD?**

A. Scrapie and chronic wasting disease
B. The consumption of "bush meat"
C. Coinfection with coxsackievirus
D. Wider consumption of meat by children

## 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 |

# Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 70% passing score) 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 may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

## Article Title

## Pretransplant Fecal Carriage of Extended-Spectrum β-Lactamase–producing *Enterobacteriaceae* and Infection after Liver Transplant, France

## CME Questions

**1. Your patient is a 62-year-old male about to undergo liver transplantation. Based on the 10-year study by Dr. Bert and colleagues, which of the following statements about incidence and characteristics of extended-spectrum β-lactamase–producing *Enterobacteriaceae* (ESBLE) infections following liver transplantation is most likely correct?**

A. Incidence of ESBLE infection within 4 months of surgery remained stable over the study period
B. During the study period, overall incidence of ESBLE infection within 4 months of liver transplantation was 5.5%
C. *E. cloacae* was the predominant causative agent
D. The median time from liver transplantation to ESBLE infection was 5 days

**2. The patient described in question 1 develops ESBLE infection after liver transplantation. Based on the 10-year study by Dr. Bert and colleagues, which of the following statements about clinical outcomes of ESBLE infection is most likely correct?**

A. Respiratory tract is the most likely site of ESBLE infection
B. Bacteremia occurs in about half of patients with ESBLE infection
C. In-hospital mortality rate is about 28%
D. About one quarter of ESBLE infections are resistant to imipenem

**3. Based on the 10-year study by Dr. Bert and colleagues, which of the following statements about risk factors for ESBLE infections following liver transplantation would most likely be correct?**

A. About one quarter of patients with ESBLE pretransplant fecal carriage developed ESBLE infections following liver transplantation
B. Independent predictors of ESBLE infections following liver transplantation were pretransplant ESBLE fecal carriage, MELD score ≥25, and return to surgery
C. Risk of ESBLE infections following liver transplantation was 5-fold higher in patients with pretransplant ESBLE fecal carriage
D. In about half of patients with pretransplant ESBLE fecal carriage and posttransplant ESBLE infection, the rectal isolate and the infecting isolate had identical patterns on ERIC-2 PCR typing

## Activity Evaluation

**1. The activity supported the learning objectives.**

| Strongly Disagree | | | | Strongly Agree |
|---|---|---|---|---|
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**2. The material was organized clearly for learning to occur.**

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|---|---|---|---|---|
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**3. The content learned from this activity will impact my practice.**

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**4. The activity was presented objectively and free of commercial bias.**

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## Article Title

## Trends in Invasive Infection with Methicillin-Resistant *Staphylococcus aureus*, Connecticut, USA, 2001–2010

## CME Questions

**1. What was the most common classification of methicillin-resistant *Staphylococcus aureus* (MRSA) over the 10-year study period of the current research?**

A. Hospital onset (HO)
B. Health care-associated community onset (HACO)
C. Community-associated (CA)
D. Undefined

**2. MRSA was most common among which of the following patient groups?**

A. Younger patients
B. Non-Hispanic black patients
C. Hispanic patients
D. Residents of small towns

**3. Which classification of MRSA was the only one to increase between 2001 and 2010?**

A. All MRSA cases
B. HO
C. HACO
D. CA

**4. Which of the following statements regarding the bacteriology of MRSA infections in the present study is most accurate?**

A. Blood isolates of MRSA were the most common infection
B. Joint isolates were more likely in HACO vs CA cases of MRSA
C. Most PFGE types were community strains of MRSA
D. The prevalence of community pulsed-field gel electrophoresis (PFGE) types declined between 2005 and 2010

## Activity Evaluation

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# EMERGING INFECTIOUS DISEASES

## Upcoming Issue

Bacterial Phenotype Variants in Group B Streptococcal Toxic Shock Syndrome

Assessment of Public Health Events through International Health Regulations, United States, 2007–2011

Implementation of International Health Regulations

Lessons Learned from Influenza A(H1N1)pdm09 Pandemic Response in Thailand

Evaluation of Control Measures for Contacts of Patient with Marburg Hemorrhagic Fever

Validity of International Health Regulations in Reporting Emerging Infectious Diseases

Adenoviruses in Fecal Samples from Asymptomatic Rhesus Macaques in Primate Facilities, United States

*Enterococcus faecalis* Clones in Poultry and Humans with Urinary Tract Infections

Differences in Spike Protein Fusion Peptide and Correlation with Feline Coronavirus Virulence

Loss of Protection from Insecticide-Treated Bed Nets against Pyrethroid-Resistant Mosquitoes, Benin

Schmallenberg Virus Antibodies among Dairy Cattle, the Netherlands, Winter 2011–2012

Predicting Risk for Death from Methicillin-Resistant *Staphylococcus aureus* Bacteremia

Migratory Birds as Reservoirs for Tick-borne Pathogens, Spain

Human Infection from Avian-like Influenza (H1N1) Viruses in Pigs, China

Changing Socioeconomic Indicators of Human Plague, New Mexico, USA

Transmission of *Bordetella holmesii* during Pertussis Outbreak, Japan

Detection of Human Calicivirus in Novel Genogroup, Bangladesh

Disseminated Microsporidiosis in an Immunosuppressed Patient

**Complete list of articles in the July issue at http://www.cdc.gov/eid/upcoming.htm**

## Upcoming Infectious Disease Activities

**June 13–16, 2012**
15th International Congress on Infectious Diseases (ICID)
Bangkok, Thailand
http://www.isid.org/15th_icid

**July 22–27 2012**
XIX International AIDS Conference (AIDS 2012)
Washington, DC, USA
http://www.aids2012.org/Default.aspx

**August 25–29, 2012**
2012 Infectious Disease Board Review Course
Ritz-Carlton, Tysons Corner
McLean, VA, USA
http://www.IDBoardReview.com

**September 9–14, 2012**
XVIIIth International Pathogenic Neisseria Conference (IPNC) 2012
Maritim Hotel, Würzburg, Germany
http://www.ipnc2012.de

**October 17–21, 2012**
IDWeek Annual Meeting
San Diego, CA, USA
http://www.IDWeek.org

**October 27–31, 2012**
APHA 140th Annual Meeting & Expo
San Francisco, CA, USA
http://www.apha.org/meetings/AnnualMeeting

### Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). 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®

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May 2012

Emerging Viruses

Collection of the Salvador Dalí Museum, Inc.; St. Petersburg, FL, USA, 2011

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

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.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 (http://wwwnc.cdc.gov/eid/pages/translations.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.** Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

**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.

**Etymologia.** Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

**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 (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. 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 of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.