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On the Cover: Tametomo no bui toukigami wo shirizoku zu

Ukiyo-e woodcut print by Yoshitoshi, c. 1890

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Anthrax of the Gastrointestinal Tract

Thira Sirisanthana* and Arthur E. Brown†

When swallowed, anthrax spores may cause lesions from the oral cavity to the cecum. Gastrointestinal anthrax is greatly underreported in rural disease-endemic areas of the world. The apparent paucity of this form of anthrax reflects the lack of facilities able to make the diagnosis in these areas. The spectrum of disease, ranging from subclinical infection to death, has not been fully recognized. In some community-based studies, cases of gastrointestinal anthrax outnumbered those of cutaneous anthrax. The oropharyngeal variant, in particular, is unfamiliar to most physicians. The clinical features of oropharyngeal anthrax include fever and toxemia, inflammatory lesion(s) in the oral cavity or oropharynx, enlargement of cervical lymph nodes associated with edema of the soft tissue of the cervical area, and a high case-fatality rate. Awareness of gastrointestinal anthrax in a differential diagnosis remains important in anthrax-endemic areas but now also in settings of possible bioterrorism.

The epidemiology of human anthrax has been described as agricultural and industrial (1). In the agricultural setting, infections occur from exposure to *Bacillus anthracis* spores on the skin or the mucosal surfaces of the gastrointestinal (GI) tract. Primary infections of the respiratory tract are rare in agricultural settings. Generally, reports state that the cutaneous form of anthrax is much more common than the GI form (2,3). We propose that the apparently overwhelming predominance of the cutaneous form of anthrax is rather a reflection of the difficulty of diagnosis of the GI form.

GI anthrax may be diagnosed on the basis of epidemiology or microbiologic, pathologic, or serologic testing. Serologic diagnosis is available only in a few research laboratories; pathologic evaluation requires surgery or necropsy in an appropriate hospital; microbiologic testing requires at least microscopy and preferably bacterial culture capacity; and epidemiology requires a level of suspicion and an ability to properly perform outbreak investigations. Herbivores, which provide most of the human exposure risk for anthrax, become infected in rural parts of the world where spores in the soil perpetuate endemicity. Mild cases of gastroenteritis attract little attention, and people with severe infections, leading to death within 2–3 days, may never reach a medical facility.

Areas endemic for anthrax exist in all continents containing tropical and subtemperate regions. In the English published reports, deaths from GI anthrax have been reported in Thailand (4–6), India (7,8), Iran (9–11), Gambia (12), and Uganda (13). Anthrax-contaminated beef from a locally infected cow was eaten in Minnesota in 2000. Cooking the meat may have prevented human cases (14). Despite this wide distribution of endemicity, no large series of pathologically described cases exists. Based on limited reports of GI anthrax, the disease spectrum ranges from the asymptomatic to the fatal, by shock or sepsis. When swallowed, anthrax spores may cause lesions from the oral cavity to the cecum. Ulcer-ative lesions, usually multiple and superficial, may occur in the stomach, sometimes in association with similar lesions of the esophagus and jejunum (4,5,15). These ulcerative lesions may bleed; hemorrhaging in severe cases may be massive and fatal (4,5).

Reported cases indicate that lesions farther down the GI tract, in the mid-jejunum, terminal ilium, or cecum, tend to develop around a single site or a few sites of ulceration and edema, more analogous to cutaneous lesions. These lesions may lead to hemorrhage (6,16), obstruction (10,17,18), perforation (17,18), or any combination of these. Ascites may complicate GI anthrax (6,7,9,11,18,19). In some patients, the fluid shift from the vascular compartment leads to shock and death (6,7,9).

The pathologic examination of anthrax lesions with entry via the GI tract shows that the mucosa is always involved, as are regional lymph nodes, which are enlarged and hemorrhagic (15). The GI tract may also be involved after disseminated infection in pulmonary and sometimes cutaneous cases (20–22). In this situation, the localization is in the submucosa as a result of its blood flow, and the mucosa and regional lymph nodes become involved only secondarily.

These reports are biased toward the hospitalized patients with severe cases. Thorough epidemiologic reports are scarce. One informative description of an outbreak investigation and response comes from Uganda (13). Gastroenteritis developed and death occurred in 155 persons who feasted on an infected zebu (Asian ox); 2 days after exposure, the incident was reported to authorities who flew in a multiministry team the next day. Gastroenteritis developed in most (92%) of those exposed within 15–72 hours. All nine deaths were in children and occurred in the first 2 days; all 12 asymptomatic cases were in adults. Authorities referred 134 symptomatic people to the hospital for rehydration and treatment with antibiotics; all recovered. Thus, for most people exposed, the syndrome was gastroenteritis; a differential diagnosis would not normally have included anthrax. The age differences suggest that in this setting previous exposure may have occurred, leaving some adults with partial immunity.

*Chiang Mai University, Chiang Mai, Thailand; and †Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand

Another community anthrax outbreak of note was investigated by officers from the Field Epidemiology Training Program of the Thai Ministry of Public Health (23). From January through June 1982, an outbreak of human anthrax was recognized in two districts in Udon Thani Province in northeastern Thailand after an outbreak in cattle that killed 36 water buffalos (*Bubalus bubalus*) and 7 cows and bulls. Of the 102 patients, 28 had cutaneous anthrax and 74 gastrointestinal anthrax. The only symptom in 67 of these 74 patients with gastrointestinal anthrax was acute diarrhea (i.e., gastroenteritis). The other seven patients had additional symptoms of nausea, vomiting, abdominal distention, and severe abdominal pain. Three patients died; the case-fatality rate was 4%. Thus, in these two community-based studies, the number of patients with GI anthrax far outnumbered those with the cutaneous form of the disease.

In addition to lack of epidemiologic, microbiologic, pathologic, and serologic expertise and facilities in the rural settings where anthrax outbreaks take place, the oropharyngeal form is underreported because few physicians are aware of the disease. Only six publications in the MEDLINE database report anthrax lesions in the mouth or oropharynx (24–29). Davies described a major epidemic of anthrax involving >9,000 patients in Zimbabwe from 1978 to 1980 (24). He cited four cases in which lesions were on the tonsil and the tongue but provided no details. We reported an outbreak of human anthrax after anthrax was found in water buffaloes in March–April 1982 in Chiang Mai, northern Thailand (25). A total of 52 cases of cutaneous anthrax and 24 cases of oropharyngeal anthrax were recognized in humans. All patients with oropharyngeal anthrax had recently eaten water buffalo meat. The mean incubation period was 42 hours (range 2–144 hours). All but one patient were admitted to the hospital. All patients sought medical attention because of painful neck swelling, and all but one complained of fever.

The other common symptoms were sore throat, dysphagia, and hoarseness. The neck swelling was usually marked and was caused by enlargement of cervical lymph nodes and soft tissue edema (Figure 1). Mouth lesions were located on the tonsils, posterior pharyngeal wall, or the hard palate. In severe cases, the tonsillar lesions extended to involve the anterior and posterior pillars of fauces, as well as the soft palate and uvula. Early lesions were edematous and congested. By the end of the first week, central necrosis and ulceration had produced a whitish patch (Figure 2). In the second week, this patch developed into a pseudomembrane covering the ulcer (Figure 3). Diagnosis could be made by culture taken from the lesion in the mouth. A Gram-stained smear from the lesion showed numerous polymorphonuclear leukocytes and gram-positive bacilli. Studies of serum antibody to anthrax antigens confirmed the diagnosis (27). Despite hospital admission and antibiotic treatment, 3 of the 24 patients died, for a case-fatality rate of 12.4%. In 1986, Doganay and colleagues reported six patients from Turkey with essentially the same clinical syn-

Figure 1. A 29-year-old man, 1 day after the onset of symptoms of oropharyngeal anthrax. Marked and painful swelling of the right side of the neck was present.

drome (28). The case-fatality rate in that study was 50%. The most recent case report, also from Turkey, was in 1993 (29).

When cattle die of anthrax, the bacteremia is massive, and manifestations of the infection are visible to the butcher. In some settings, people may eat meat they know to be contaminated, as was the situation in a poor village of Harijans ("untouchables") in India (8). More common may be the situation in which meat is sold to those unaware of the animal disease as was the case in the Chiang Mai outbreak (25). Only those who eat dishes that are raw or undercooked are exposed to infectious material. Disease is likely related to a dose of viable spores and the immune state of host. In contrast to the extreme susceptibility of cattle to this infection and its bacteremia, studies in chimpanzees suggest that primates are relatively resistant (30).

While the biowarfare and bioterrorist development of anthrax has focused on inhalation, ingestion has been considered as well. The Japanese experiments in China during the 1930s and 1940s included attempts to poison children with chocolate impregnated with anthrax (31). More recently, the apartheid government of South Africa had developed biological weapons, including another attempt at anthrax-containing

Figure 2. A 27-year-old man, 5 days after the onset of symptoms of oropharyngeal anthrax. Edema and congestion of the right tonsil extending to the anterior and posterior pillars of fauces as well as the soft palate and uvula were present. A white patch had begun to appear at the center of the lesion.

Figure 3. The same patient as in Figure 2. This picture is 9 days after the onset of symptoms of oropharyngeal anthrax. The white patch had developed into a pseudomembrane covering the lesion.

chocolate (32). Given the large community outbreak of salmonellosis caused by an intentional contamination of restaurant salad bars the in United States by the Rajneeshees (33), awareness of the potential for GI anthrax due to bioterrorism is important.

In conclusion, GI anthrax is probably greatly underreported in rural disease-endemic areas of the world. The spectrum of disease, ranging from no symptoms to death, has not been fully appreciated. Awareness of anthrax in a differential diagnosis remains important in disease-endemic areas and also in settings of possible bioterrorism.

Dr. Sirisanthana is professor of medicine and director of the Research Insitute for Health Sciences, Chiang Mai University, Thailand. He received his medical degree from Mahidol University in Bangkok and then trained in internal medicine in infectious disease at Indiana University, the University of Chicago and the Medical College of Wisconsin from 1975 to 1979.

LTC Brown is chief, Department of Retrovirology, at the Armed Forces Research Institute for Medical Sciences in Bangkok. He is a fellow of the Infectious Disease Society of American with training in tropical public health. Dr. Brown's research in tropical infectious diseases has been based largely in Thailand for the past 20 years.

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Address for correspondence: Arthur E. Brown, AFRIMS, APO AP 96546; fax: 66-02-644-4824; e-mail: brownae@thai.amedd.army.mil

Emergence of *Usutu virus*, an African Mosquito-Borne *Flavivirus* of the Japanese Encephalitis Virus Group, Central Europe

**Herbert Weissenböck,* Jolanta Kolodziejek,† Angelika Url,* Helga Lussy,†
Barbara Rebel-Bauder,* and Norbert Nowotny†‡**

During late summer 2001 in Austria, a series of deaths in several species of birds occurred, similar to the beginning of the *West Nile virus* (WNV) epidemic in the United States. We necropsied the dead birds and examined them by various methods; pathologic and immunohistologic investigations suggested a WNV infection. Subsequently, the virus was isolated, identified, partially sequenced, and subjected to phylogenetic analysis. The isolates exhibited 97% identity to *Usutu virus* (USUV), a mosquito-borne *Flavivirus* of the Japanese encephalitis virus group; USUV has never previously been observed outside Africa nor associated with fatal disease in animals or humans. If established in central Europe, this virus may have considerable effects on avian populations; whether USUV has the potential to cause severe human disease is unknown.

*U*sutu virus (USUV) is a relatively unknown member of the mosquito-borne cluster within the *Flavivirus* genus, closely related to important human pathogens such as *Japanese encephalitis virus* (JEV), *Murray Valley encephalitis virus* (MVEV), *Dengue virus* (DENV), *Yellow fever virus* (YFV), *Saint Louis encephalitis virus* (SLEV), and *West Nile virus* (WNV) (1–3). Isolated for the first time from mosquitoes in South Africa in 1959 and named after a river in Swaziland (4), USUV was sporadically isolated from several mosquito and bird species over the next decades (5–7). Only two isolations have been reported from mammals, one from *Praomys* sp. (African soft-furred rats) and one from a man with fever and rash (5). The virus has never been associated with severe or fatal diseases in animals or humans, and it has never before been observed outside tropical and subtropical Africa.

From the beginning of August through mid-September 2001, a considerable die-off of Eurasian Blackbirds (*Turdus merula*) was observed in and around Vienna, Austria. Some observers reported obviously sick blackbirds, which showed signs of apathy and ruffled plumage. Within 5 days in mid-August, five Great Gray Owls (*Strix nebulosa*) died in the Tiergarten Schönbrunn Vienna Zoo. In addition, many dead Barn Swallows (*Hirundo rustica*) were observed in the Austrian federal state of Upper Austria, 200 km west of Vienna. Investigating this episode of avian deaths in Austria, we determined USUV as the causative agent.

Methods

In total, we received six blackbirds, five owls, and one swallow for investigation. At necropsy (estimated postmortem times between 24 h and 48 h), tissue samples were fixed in 7% buffered formalin. After embedding in paraffin wax, 4-µm sections were stained with hematoxylin and eosin.

Paraffin-embedded tissue samples were immunostained with a polyclonal mouse antibody to WNV (B. Murgue, Institut Pasteur, Paris) and a polyclonal rabbit antibody to *Tick-borne encephalitis virus* ([TBEV] strain Neudoerfl, H. Holzmann, Klinisches Institut für Virologie, Vienna) using the Avidin-Biotin Complex technique (8).

RNA was extracted from 140-µL organ homogenates or cell culture suspensions by using the QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany). After aligning available nucleotide (nt) sequences of various mosquito-borne flaviviruses and determining highly conserved genomic regions, we designed three pairs of oligonucleotide primers (to amplify a wide range of mosquito-borne flaviviruses) and used them in the reverse transcription-polymerase chain reaction (RT-PCR) assays: 5'-TACAACATGATGGGVAARAGAGAGA-3' (nt position 9031–9055 of WNV GenBank accession no. NC 001563) and 5'-AGCATGTCTTCYGTBGTCATCCAYT- 3' (nt position 10115–10091), resulting in a 1,084-bp amplification product; 5'-GARTGGATGACVACRGAAGACATGCT-3' (nt position 10090–10115) and 5'-GGGGTCTCCTCTAACCTC TAGTCCTT-3' (nt position 10832–10807), amplifying a 743-bp PCR product; and 5'- GCCACCGGAAGTTGAGTAGA-3' (nt position 10460–10479 of WNV no. NC 001563) and 5'-GCTGGTTGTGCAGAGCAGAA-3' (nt position 10908–10889), resulting in a 449-bp amplicon. Reverse transcription and amplifications were performed in a continuous RT-PCR

method by using the QIAGEN OneStep RT-PCR Kit (Qiagen GmbH). Each 25-µL reaction mixture contained 5 µL 5X buffer (final MgCl2 concentration 2.5 mM), 0.4 mM deoxynucleoside triphosphate (dNTP), 10 U recombinant RNasin Ribonuclease Inhibitor (Promega, Madison, WI), 40 pmol forward and reverse primers, 1 µL enzyme mix, and 2.5 µL template RNA. Reverse transcription was performed for 30 min at 50°C. Following an initial denaturation for 15 min at 95°C, the reaction mixture was subjected to 45 cycles of heat denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and DNA extension at 72°C for 1 min, completed by a final extension of 10 min at 72°C. Following RT-PCR, we performed electrophoresis on 20 µL of the amplicons in a 1.2% Tris acetate- EDTA-agarose gel. The gel was stained with ethidium bromide, and the bands were observed under UV light.

We excised the fragments from gel, extracted DNA, and performed sequencing PCR. The PCR products were sequenced in both directions by using the ABI Prism 310 genetic analyzer automated sequencing system (Perkin Elmer Instruments, Wellesley, MA). The nucleotide sequences were compiled and aligned with the corresponding sequences deposited in the GenBank database. Finally, we constructed a phylogenetic tree based on a 1,035-nt fragment in the NS5 genomic region. The following sequences have been included in the phylogenetic analysis: AF013384, *Koutango virus*; AF013413, *Yaounde virus*; D00246, Kunjin virus; AF202541, WNV (New York 1999); M12294, WNV; AF013367, *Cacipacore virus*; U15763, JEV; AF013360, Alfuy virus; AF013389,

MVEV; AF013412, USUV (South Africa); AF452643, USUV (Austria); AF013416, SLEV; M93130, DENV (type 3); and AF013417, YFV. The phylogenetic analysis was carried out by using Phylogeny Interference Program Package (PHYLIP), version 3.57c (available from: URL: http://evolution.genetics.washington.edu/phylip.html). We generated bootstrap resampling analysis of 100 replicates with the SEQBOOT program. Distance matrices were generated with the DNADIST/ Neighbor-Joining program; a translation/transversion ratio of 2.0. AF013417 (YFV) was used as outgroup.

Paraffin-embedded tissue samples were processed as described (9). For detection of WNV nucleic acid, an antisense digoxigenin- labeled riboprobe complementary to nt 4966–5439 of WNV strain NY1999 was generated from plasmid pWNNY-88B-14 (W.I. Lipkin, Emerging Diseases Laboratory, Irvine, CA). The final concentration of the probe was approximately 0.5 ng/µL. For detection of USUV nucleic acid, we used a digoxigenin-labeled oligonucleotide probe with the sequence: 5'- TCGCATAACTTTCACCACCTTGTGTTTGTA GGTCAGCTC-3', which is complementary to nt 367-328 of the accessible partial sequence of the NS5 gene of USUV (GenBank accession no. AF013412). The final probe concentration was approximately 0.25 ng/µL.

Results

Necropsy showed grossly swollen livers and spleens, as well as seromucous enteritis in all blackbirds and owls; histology showed various degrees of multifocal acute necrosis in liver and spleen. Although the blackbirds did not show
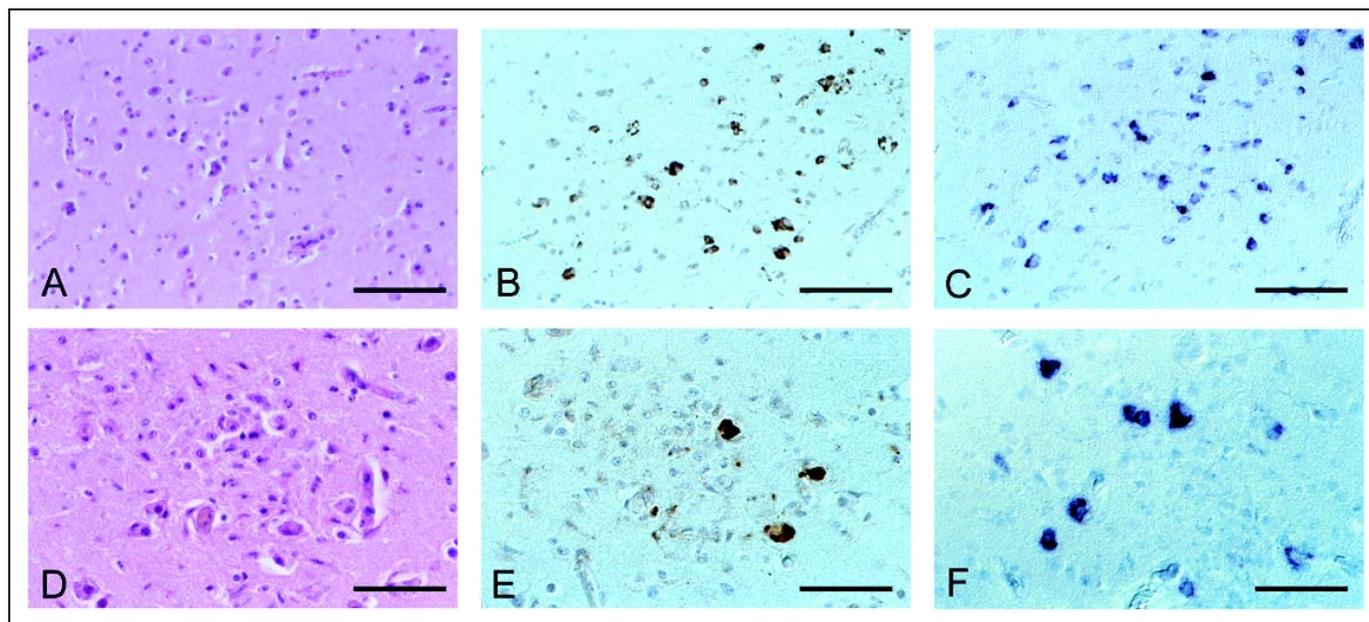
Figure 1. Histology and detection of viral signals in paraffin-embedded tissue sections of birds infected with *Usutu virus* (USUV). A–C, Eurasian Blackbird; D–F, Great Gray Owl; A, no histologic lesions present, hematoxylin and eosin staining; B, immunohistochemistry, using a polyclonal antibody to *West Nile virus*, shows numerous positive neurons; C, in situ hybridization with USUV-specific oligonucleotide probe shows a staining pattern comparable with that in B; D, microglial nodule within the cerebral cortex, hematoxylin and eosin staining; E, immunohistochemistry shows single positive neurons within a glial nodule; F, in situ hybridization shows several positive neurons next to a glial nodule. Bar = 75µm (A–C); 50µm (D–F).

obvious histologic brain lesions, the owls had encephalitis, predominantly shown as multifocal areas of neuronophagia and microgliosis (Figure 1, A and D). Pathohistologic and immunohistochemical investigation of the swallow did not yield useful results because of severe autolysis.

Immunohistochemistry (IHC) with polyclonal antibodies to WNV was positive in 10 of 11 brains, showing reaction products in neurons and their processes and in the cytoplasm of microglial cells in glial nodules (Figure 1, B and E). Positive reactions were also present in kidney, spleen, liver, lung, and autonomous ganglia of the gastrointestinal tract. Brain and kidney samples from WNV-infected birds from the United States and Israel, respectively, were positive controls; blackbirds that died from trauma were negative controls. IHC with polyclonal antibodies to TBEV, another flavivirus found in central Europe, showed negative results. RT-PCR with WNV-specific primers and ISH with a WNV-specific probe were negative. Infection with a flavivirus related to WNV would account for the cross-reactivity of a polyclonal antibody used in IHC and the negative outcome of the WNV-specific assays.

Organ homogenates of blackbirds and owls were added to Vero cell cultures. After 24–48 hours, a cytopathic effect of cell rounding could be observed; 1 to 2 days later, the affected cells detached and floated in the medium.

RT-PCRs with universal flavivirus primers resulted in clear PCR amplification products of the expected lengths. The primers were designed to amplify overlapping PCR products in the NS5 genomic region of mosquito-borne flaviviruses. RT-PCRs were performed both on the original organ homogenates and on cell culture suspensions, with identical results. Despite a poor state of preservation, organ homogenates of the swallow also proved positive by RT-PCR.

The PCR products (1,084 bp, 743 bp, and 449 bp) were directly sequenced in both directions; the compiled nucleotide sequences (a stretch of 1,877 bp, representing approximately 17% of flavivirus genome) were aligned and compared with other sequences by using the Basic Local Alignment Search Tool (BLAST) search (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD). The sequence obtained from the Austrian dead birds was 97% identical with a 1,035-nt fragment of USUV (GenBank accession no. AF013412; [2]), an African mosquito-borne flavivirus in the JEV group. To investigate the genetic relationship of the Austrian USUV with other flaviviruses in the JEV antigenic complex, we constructed a phylogenetic tree by using the programs in the PHYLIP package; we also included in the phylogenetic analysis three other important mosquito-borne flaviviruses (YFV, SLEV, and DENV3). The phylogenetic tree (Figure 2) demonstrates the close genetic relationship between USUVs isolated in South Africa and in Austria; therefore, we classified the Austrian USUV as part of the JEV group of flaviviruses. At the amino-acid level, the Austrian and the South African USUV isolates proved (in the investigated 1,035-nt region) to be 100% identical.
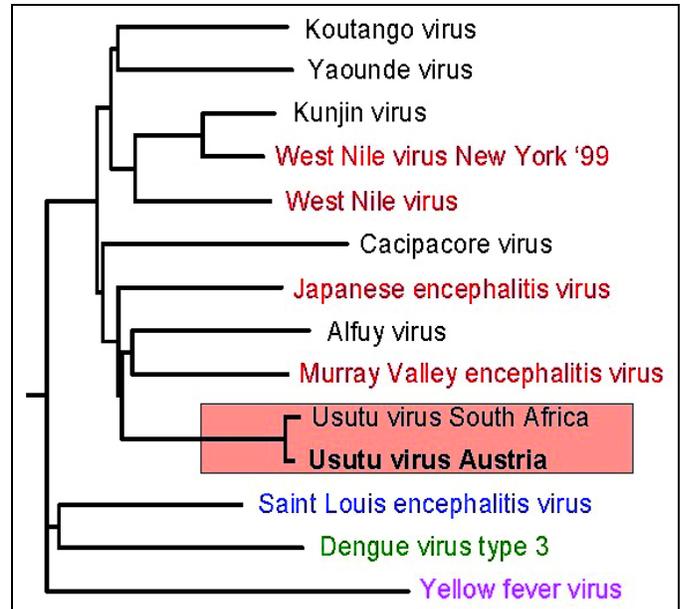
Figure 2. Phylogenetic analysis of several members of the *Japanese encephalitis virus* (JEV) group and selected other mosquito-borne flaviviruses demonstrates the close genetic relationship of the Austrian *Usutu virus* (USUV) isolate with the South African USUV (red underlay); well-known members of the JEV group are highlighted in red; distinct branches are formed by *Saint Louis encephalitis virus*, *Dengue virus* (type 3), and *Yellow fever virus*. The partial nucleotide sequence of the Austrian USUV isolate used in the phylogenetic tree has been deposited in the GenBank database under accession no. AF452643.

With an oligonucleotide probe specific for USUV, ISH showed presence of viral nucleic acid in the cytoplasm of neurons in a distribution pattern closely matching IHC (Figure 1, C and F). Regarding other organs, however, kidneys of only two birds were positive, probably reflecting RNA degradation due to postmortem times >24 h.

Discussion

We demonstrated the presence of a mosquito-borne flavivirus, never before observed outside tropical and subtropical Africa, in the continental climate of central Europe, where winter temperatures are as low as -20°C. Since we also detected USUV nucleic acid in a Barn Swallow, the virus was probably introduced to the Austrian bird population by swallows or other migrating birds. Bird die-offs in various bird species in different areas of Austria suggest that the virus has already adapted to local mosquito species, which are probably transmitting the virus. Isolating the virus from local mosquitoes has not been attempted thus far but is planned. During a retrospective survey of paraffin-embedded blackbird tissues by IHC and RT-PCR, we also detected USUV in a blackbird that died a year earlier (2000). A partial nucleotide sequence of this USUV proved to be 100% identical to the sequences of the 2001 USUV isolates. Although no severe bird die-offs were observed in 2000, we think that USUV may already be established and be overwintering in Austria (rather than being newly introduced in 2 consecutive years). Comparable with the introduction of WNV to North America in 1999, where the

virus propagated in local mosquito species and then rapidly spread from New York to >20 states in the United States and Canada (10), we foresee a similar scenario for USUV in Europe.

This study shows for the first time that USUV is highly pathogenic for several different species of birds. Because full sequence data of USUV isolates are not available (only a short [1,035-bp] fragment of one USUV isolate has been deposited in the GenBank database), we cannot fully compare the strain isolated here with the African isolates. We cannot provide information on amino acid changes that might contribute to altered pathogenic properties. For the closely related WNV, the pathogenicity for birds seems to depend on the virus strain and whether the virus affects a previously exposed or unexposed population. The WNV strain that appeared in North American birds in 1999 is closely related to a strain isolated in Israel; this strain is associated with avian deaths in both countries (11–14). Certain other WNV strains, such as those responsible for recent outbreaks in Romania and Russia in humans (15,16) and in Italy and France in equines (17,18), were not associated with avian deaths. Also, the fact that certain avian species, such as Eurasian Blackbirds, Great Gray Owls, and Barn Swallows in Austria, are especially vulnerable to USUV infection, is reminiscent of the observation that WNV in North America has primarily affected American Crows and Blue Jays (19,20).

The emergence of WNV in the United States in 1999 and USUV in central Europe in 2000–2001 is an indication of future virus activity. The next mosquito-borne flavivirus, which might be introduced to regions far from its original habitats, may be highly pathogenic for humans, farm animals, or pets, as many strains of JEV group are. We could speculate whether global warming or other environmental factors may have contributed to the introduction and maintenance of USUV, formerly restricted to tropical and subtropical areas, in a much colder climate. As a consequence of the introduction of USUV to Central Europe, surveillance programs for mosquito-borne flaviviruses in general (based on virus detection in mosquitoes and dead birds, as well as epidemiologic investigations) should be established in Europe, like those initiated in the United States after the first occurrence of WNV (19,20). Moreover, we will have to fully sequence USUV, establish serologic test systems, and evaluate the spread and pathogenic potential to control this new virus infection in Central Europe.

Dr. Weissenböck is an associate professor at the University of Veterinary Medicine, Vienna. His research interests include neuropathology, neurovirology, and molecular pathology.

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Address for correspondence: Norbert Nowotny, Clinical Virology Group, Institute of Virology, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria; fax: 43 1 25077 2790; e-mail: Norbert.Nowotny@vu-wien.ac.at

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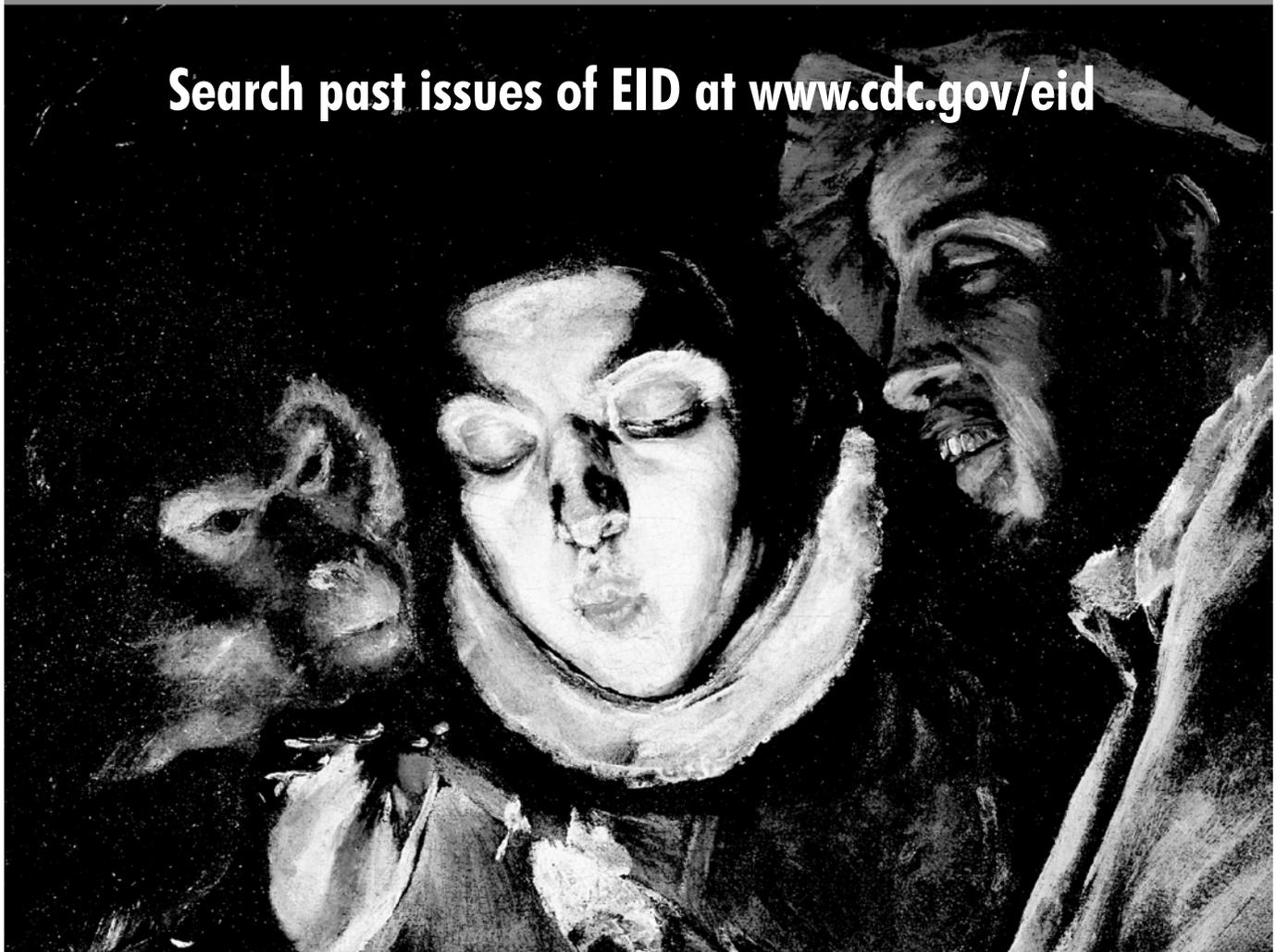
First Human Isolate of Hantavirus (*Andes virus*) in the Americas

Hector Galeno,* Judith Mora,* Eliecer Villagra,* Jorge Fernandez,* Jury Hernandez,†
Gregory J. Mertz,‡ and Eugenio Ramirez*

We isolated Andes virus (formal name: *Andes virus* [ANDV], a species in the genus *Hantavirus*), from serum of an asymptomatic 10-year-old Chilean boy who died 6 days later of hantavirus pulmonary syndrome (HPS). The serum was obtained 12 days after his grandmother died from HPS and 2 days before he became febrile. No hantavirus immunoglobulin (Ig) G or IgM antibodies were detected in the serum sample. After three blind passages, ANDV antigens were detected in Vero E6 cells by immunofluorescence assay and enzyme-linked immunosorbent assay, and ANDV RNA was detected by reverse transcription-polymerase chain reaction. A fragment of the virus genome showed 96.2% nucleotide identity with that of prototype ANDV. To our knowledge, this is the first isolation of any agent of hemorrhagic fever with HPS from a human and the first such isolation of hantavirus before symptoms of that syndrome or HPS began.

Hantaviruses are rodent-borne negative-sense RNA viruses that cause hemorrhagic fever with renal syndrome or hantavirus pulmonary syndrome (HPS) in humans (1). HPS is caused by New World hantaviruses that have been identified since the syndrome was first recognized in the southwestern United States in 1993 (2). The syndrome is characterized by four stages: the febrile prodrome, the cardiopulmonary stage, diuresis, and convalescence. The cardiopulmonary phase, which typically lasts 2–10 days, can range from a mild illness, characterized by shortness of breath and need for supplemental nasal oxygen, to severe cardiopulmonary involvement with respiratory failure, lactic acidosis, shock, and death (3,4). In survivors, diuresis occurs abruptly and is usually associated with rapid clinical improvement. The course and duration of the convalescent stage are more variable, but most patients describe persistent fatigue and limited tolerance for exercise for at least several months.

HPS has been reported in Argentina, Brazil, Canada, Chile, Panama, Paraguay, Uruguay, and the United States; mortality rates range from 40% to 50% (1). In Chile, 135 cases of HPS have been reported through February 9, 2001, with a 48.8% mortality rate (5). Andes virus (formal name: *Andes virus* [ANDV], a species in the genus *Hantavirus),* which is carried by *Oligoryzomys longicaudatus,* is responsible for most HPS cases in Argentina and Chile. In contrast, Sin Nombre virus (formal name: *Sin Nombre virus* [SNV]), which is carried by *Peromyscus maniculatus,* is the primary pathogen in North America. No evidence has been found to support person-to-person transmission of SNV, but person-to-person transmission of ANDV has been documented in one large outbreak in Argentina (6) and is suggested by case clustering in

household contacts in Chile (M. Ferres, X. Aguilera, pers. comm.).

Most patients are seen at the onset of the cardiopulmonary phase, and information about clinical and laboratory findings, viremia, and immune responses is most complete for this and subsequent phases (7,8). Less is known about clinical and laboratory findings, viremia, and immune responses during the febrile prodrome, although both specific immunoglobulin (Ig) G and IgM antibodies are almost always present during this phase (9). In contrast, no information is available on the development or time course of viremia or immune responses before symptoms begin (in the prodromal phase). We describe the first isolation of hantavirus from a human in the Americas and the first isolation of hantavirus from a human before onset of symptoms of HPS or hemorrhagic fever with renal syndrome.

Patients and Methods

Case Descriptions

The index patient was a 54-year-old woman who had headache, myalgias, and abdominal pain on August 26, 1999, followed several days later by respiratory symptoms. She went to the hospital on August 31, where she was diagnosed with bilateral pneumonia and adult respiratory distress syndrome; she died on September 1. A serum sample obtained on August 31 was reactive for IgM antibodies. The patient's 71-year-old brother had had a febrile illness on August 7, 1999, and was hospitalized 2 days later with a clinical diagnosis of acute abdominal pain**,** pyelonephritis, shock, and bilateral pulmonary infiltrates; he died on August 10. HPS was not suspected, and no serum or tissue was available for testing when HPS was diagnosed in the index patient.

The Ministry of Health initiated a routine evaluation of household and neighborhood contacts on September 13, 1999. Blood was obtained from 10 asymptomatic contacts,

including the 10-year-old grandson of the index patient. On September 15, the grandson became febrile, and headache and vomiting developed. Two days later, he (patient 99-7913) was evaluated as an outpatient. His physical examination showed fever (38°C) and no respiratory symptoms. His leukocyte count was 13,000/μL, hematocrit 46.9%, hemoglobin 15.7 g/dL, and platelet count 125,000/μL. The plasma C reactive protein was 39 mg/L. Diffuse bilateral interstitial pulmonary infiltrates were detected on chest radiograms, and the patient was treated with a macrolide antibiotic for presumed pneumonia. He returned to the hospital the morning of September 18 without fever, with arterial pressure 110/60 mmHg, tachycardia (100 beats per minute), and weakness. Pneumonia, obstructive bronchial syndrome, and dehydration were diagnosed. He was treated with intravenous penicillin, hydration, and aerosolized salbutamol. He returned to the hospital again on the evening of September 18 with respiratory failure and shock and died on September 19 within hours of arrival. No additional serum or tissue samples were obtained at the outpatient visit or in the hospital.

Epidemiologic Studies

Routine epidemiologic evaluation of each confirmed HPS case in Chile includes rodent trapping around the patient's household and evaluation of household and family contacts. The latter includes a clinical evaluation for history of recent fever or other symptoms and the administration of a questionnaire to assess risk factors for hantavirus infection. A serum sample is obtained from household and family contacts by venipuncture and transported to the Institute of Public Health in Santiago for determination of hantavirus antibodies.

Biosafety Procedures

We followed the recommendations of the Centers for Disease Control and Prevention (CDC) in all aspects of this work (10). Antibody studies were conducted at biosafety level 2 (BSL-2) facilities, but viral isolation attempts were conducted at BSL-3 laboratories in the Instituto de Salud Pública laboratory in Santiago.

Detection of Antibodies to SNV in Patient Serum

We detected antibodies from patient serum samples by enzyme-linked immunosorbent assay (ELISA) with nucleocapsid (N) antigens of SNV and Laguna Negra (formal name: *Laguna Negra virus* [LNV]) virus. These diagnostic tests were obtained from CDC in Atlanta, Georgia (11). SNV antigen was used in the solid phase for detecting IgG antibodies, and LNV antigen was used in an antigen-capture format for detecting specific IgM antibodies, as described (11).

Isolation of ANDV from Patient Serum

Virus was isolated by a conventional method, with three blind passages in monolayers of Vero E6 cells (12). We grew Vero E6 cells to confluence in T25 flasks with minimal essential media containing 10% fetal calf serum and antibiotics, and

then replaced the media with 1.5 mL of fresh media containing 0.5 mL of serum from patient 99-7913. After 1 hour, we added 4.5 mL of fresh media and maintained the cells at 37°C in 5% CO_2 for 26 days, refeeding twice per week (Tissue Culture 1 or TC-1). At 26 days, the cells were treated with trypsin and split 1:2 into fresh T25 flasks (TC-2). At 24 days postinoculation (dpi), we trypsinized the cells and replated them into fresh T25 flasks (TC-3). At 14 dpi, we trypsinized the cells and replated them into fresh T25 flasks (TC-4). At 13 dpi, we treated the cells with trypsin and replated them after removing 5×10^4 for indirect immunofluorescence assay (IFA) analysis.

IFA

Fifty thousand inoculated Vero E6 cells were washed twice with phosphate-buffered saline and dried on a microscope spot-slide for IFA testing. As a control, we used uninoculated Vero E6 cells that were processed in parallel. The cells were stained according to Gallo et al. (13), using a 1:1,000 dilution of rabbit polyclonal anti-Andes N. Cells were considered to be positive for hantavirus antigen if we observed punctate cytoplasmic and Golgi staining in the presence of anti-N antibody but not in the presence of preimmune rabbit serum or if the cells had not been injected with a source of ANDV.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

We conducted nested RT-PCR to detect a portion of the viral N gene by using generic primers for S segment as described (14). The coordinates of the primers, designated S1 and S2 (outer primers), were at 2 and 593, whereas the inner primers S3 and S4 were at coordinates 22 and 359. We determined that an amplification reaction was positive if we could visualize a 338-bp band after electrophoresis through agarose, as described (14).

Phylogenetic Studies

Nucleotide sequences examined corresponded to positions 22–359 of antigenome-sense sequence of nucleoprotein gene. Genetic relationships of the Chilean isolate with homologous sequences of previously characterized hantavirus were obtained by the maximun parsimony method with the Clustal W and PHYLIP packages (15).

Results

The grandson's death from suspected HPS was reported on September 21. Serum obtained on September 13, which had been transported and stored at 4°C, was tested for the presence of hantavirus antibodies by ELISA. Neither IgG nor IgM antibody was detected. Serum was then injected into tissue Vero E6 cell monolayers to attempt viral isolation (12).

Virus Isolation

Inoculated and noninoculated Vero E6 cells were cultivated for several weeks. After three blind passages in new monolayers of Vero E6 cells, the presence of hantaviral antigens was

tested by IFA with serum samples from seropositive Chilean HPS patients. Only the cells derived from the patient serum-inoculated tissue culture expressed fluorescent inclusion bodies (Figure 1). Over 90% of the cells became fluorescent. The specificity of this reaction was further tested by ELISA with cellular lysates prepared from infected and noninfected cells and ANDV antibody-positive rabbit sera. The absorbance values obtained by using lysates of infected cells were higher than those from noninfected cells (data not shown). To further confirm the presence of hantavirus infection in the Vero E6 cells, we also used nested RT-PCR of the nucleoprotein gene to test tissue cultures inoculated with patient serum with generic primers for S segment (14). Only the RNA extract from patient sera-inoculated cells showed an amplified product with the expected 338-bp fragment. The amplified product was not detected in mock-infected cells (data not shown).

The amplified DNA product was sequenced and compared with the sequence of prototype strains (Figure 2). The Chilean hantavirus isolate, designated CHI-7913, showed 96.2% nucleotide identity with the prototypical ANDV sequence, but only 81.1%–82.5% identity with SNV from the southwestern United States (14).

Discussion

Previous human isolates of hantaviruses have been reported only for Old World viral species, and no isolates have been described from patients who were asymptomatic. Since previous attempts at other laboratories had been unsuccessful, we attempted isolation before the onset of symptoms, and our first attempt to do so was successful. Our results document the presence of infectious hantaviral virions in a serum specimen obtained from a seronegative 10-year-old child 2 days before his symptoms began and 6 days before his death from HPS. We excluded the possibility of laboratory contamination since no hantavirus was or had been in use in our laboratory nor were we making other attempts to isolate hantavirus.

Based on the partial S segment sequence we obtained, the isolate CHI-7913 is a geographic variant of ANDV. Comparison of CHI-7913 virus N gene sequence with the corresponding sequences of representative New World hantaviruses showed the highest degree of identity with that of ANDV.

Isolation of hantaviruses from rodents and humans has been difficult, and isolation from humans has been particularly so. Many apparent human isolates were obtained in laboratories that were actively cultivating a number of hantavirus strains at the time of the isolation. Thus, several earlier human isolates have proven difficult to confirm as independent isolates after they were subjected to genetic comparison with previously obtained rodent isolates (25). Other human isolates that have been reported more recently have not been subjected to similar scrutiny. In 19 attempts over more than a decade, Juto et al. reported one successful attempt at isolation of Puumala virus (formal name: *Puumala virus* [PUUV]) from phytohemagglutinin-stimulated human leukocytes (26). In a report of isolation of Hantaan virus (formal name: *Hantaan virus*
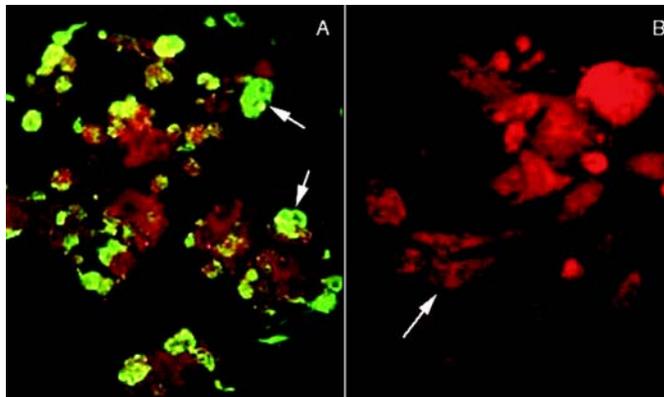
Figure 1. Immunofluorescence assay (IFA) of Vero E6 cells infected with Chilean hantavirus CHI-7913 isolate. A, IFA with seropositive human sera from a Chilean HPS patient; arrow shows infected Vero E6 cells expressing hantavirus antigens. B, IFA with seronegative human sera from uninfected control; arrow shows the negative IFA of Vero E6 cells infected with the CHI-7913 isolate.

[HTNV]) from peritoneal exudate cells collected from a patient with severe hemorrhagic fever with renal syndrome on the 10th day of illness, Gu et al. noted that human isolation of HTNV is easier from blood collected during the first 4 days of illness than from blood collected after day 6 (27). Examining these and other human isolates of PUUV and HTNV by sequence analysis will be valuable in confirming their independent origin, much as we have been able to do with CH-7913.

We suspect that development of neutralizing antibody early in symptomatic illness may be the primary factor leading to difficulty in isolation of hantavirus from blood in humans after illness has begun. Bharadwaj et al. recently reported detecting neutralizing antibody in all sera obtained at the day of hospital admission in patients with SNV-associated HPS (9). Although most hospital admissions occurred at the onset of the cardiopulmonary stage, neutralizing antibody was also detected in a limited number of sera available 1 or 2 days before hospital admission, during the prodromal phase. The recent report that viral RNA detected by RT-PCR inevitably declines early in hospitalization (7,28) is also consistent with the hypothesis that virus is present but that neutralizing antibody and other immune responses are already reducing the titer and infectiousness of hantaviruses by the cardiopulmonary stage of illness. That stage is when most patients come to medical attention.

We were able to isolate hantavirus from serum obtained 2 days before symptoms began and before the production of detectable levels of IgG or IgM antibodies. This finding suggests that a viremic phase may precede symptoms and that the onset of symptoms in the prodromal stage may be associated with humoral and cellular immune responses rather than viremia. In contrast to HPS in North America, where case clusters are uncommon, approximately one third of HPS cases in Chile have occurred in clusters involving members of the same household or other close contacts (M Ferres, X Aguilera, pers. comm.). Of these, most have involved case clusters with symptom onset separated by 2–4 weeks rather than case clus-
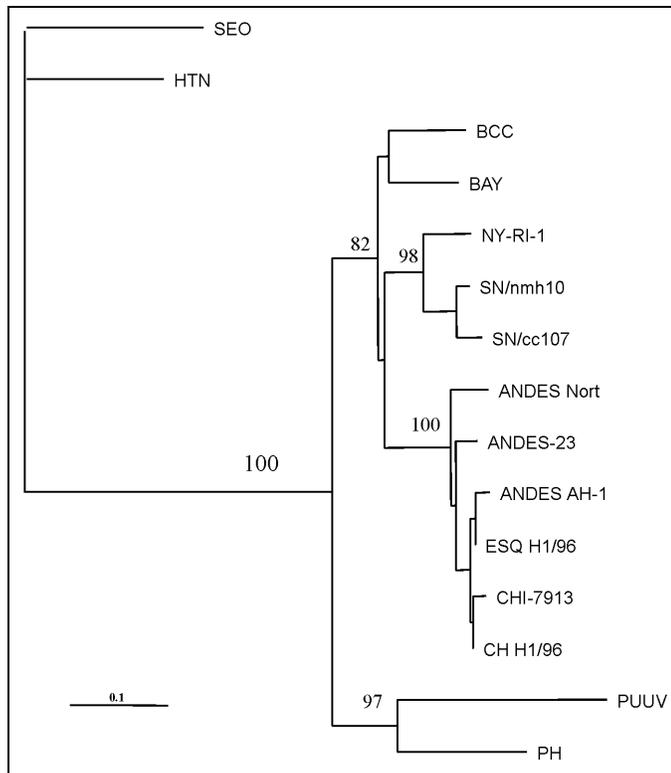
Figure 2. Maximum-parsimony tree analysis comparing S nucleotide sequence of CHI-7913 virus with homologous sequences of previously characterized hantaviruses. Nucleotide sequences examined correspond to positions 22–359 of antigenome-sense sequence of nucleoprotein (N) gene. Sequences were analyzed by the maximun parsimony method with the Clustal W and PHYLIP packages (15). The minimal length trees shown were supported as the majority rule consensus tree in 500 replicates. The bootstrap replicates supporting each node are indicated. References and GenBank accession numbers for the sequences used in S segment comparisons are BCC (16) L39949; BAY (17) L36929; NY strain RI-1 (18), U09488; SN strain cc107 (19), L33683; SN strain nmh10 (20), L25784; PH strain PH-1 (21), and M34011; Puumala strains Sotkamo (22), X61035; Seoul (SEO) strain sr-11 (23), and M34881; Hantaan (HTN) strain 76-118 (24), M14626; Andes strain AH-1 (14), AF004660; ESQ H-1/96 (14), AF005948; CH H-1/96 (14), AF 005947; AND Nort (strain unpublished) AF325966; and Andes strain 23 (AF291702).

ters with closely related dates of symptom onset. As such, Chile may pose a unique opportunity to prospectively follow close contacts of index patients to determine whether viremia routinely precedes symptoms as well as to identify and perhaps treat some persons early in the course of symptomatic hantavirus illness.

Dr. Galeno works in the hantavirus laboratory at the Public Health Institute of Chile. He is conducting research on immune responses (neutralizing antibodies) of infected patients.

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Address for correspondence: Eugenio Ramirez, Public Health Institute of Chile, Av. Marathon 1000, Santiago, Chile; fax: 562-3507573; e-mail: eramirez@ispch.cl

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Ecologic Niche Modeling and Potential Reservoirs for Chagas Disease, Mexico

A. Townsend Peterson,* Victor Sánchez-Cordero,† C. Ben Beard,‡ and Janine M. Ramsey§

Ecologic niche modeling may improve our understanding of epidemiologically relevant vector and parasite-reservoir distributions. We used this tool to identify host relationships of *Triatoma* species implicated in transmission of Chagas disease. Associations have been documented between the protracta complex (*Triatoma*: Triatominae: Reduviidae) with packrat species (*Neotoma* spp.), providing an excellent case study for the broader challenge of developing hypotheses of association. Species pairs that were identified coincided exactly with those in previous studies, suggesting that local interactions between *Triatoma* and *Neotoma* species and subspecies have implications at a geographic level. Nothing is known about sylvatic associates of *T. barberi*, which are considered the primary Chagas vector in Mexico; its geographic distribution coincided closely with that of *N. mexicana*, suggesting interaction. The presence of this species was confirmed in two regions where it had been predicted but not previously collected. This approach may help in identifying Chagas disease risk areas, planning vector-control strategies, and exploring parasite-reservoir associations for other emerging diseases.

C hagas disease is caused by the parasitic protozoan *Trypanosoma cruzi* and transmitted by blood-feeding insects in the family *Reduviidae*, subfamily Triatominae. Chagas disease is an important cause of illness and death throughout the Americas, affecting 16–18 million persons. While an estimated 100 million persons in 21 countries in the New World live in endemic areas and are at risk for infection, the disease is principally a zoonotic infection, in which sylvatic mammals serve as reservoir hosts and zoophilic triatomine species as vectors.

The protracta species group consists of seven species (*Triatoma protracta, T. peninsularis, T. sinaloensis, T. neotomae, T. barberi, T. nitida,* and *T. incrassata*); *T. protracta* contains five subspecies: *T. p. protracta, T. p. woodi, T. p. navajoensis, T. p. zacatecensis,* and *T. p. nahuatlae* (1,2). This group is restricted to the southwestern United States and Mexico. Previous studies have demonstrated high host specificity in this species group, involving woodrats or packrats (*Neotoma* spp.) (1). Whereas host associations of *Triatoma* are often complex, the protracta group shows remarkable host specificity and geographic distributions suggestive of host-ectoparasite cospeciation.

A new tool in the study of geographic phenomena in ecology and systematics is ecologic niche modeling of primary occurrence data (data placing a particular species in a particular site) (3). In general, the approach involves a machine-learning algorithm for discovering associations between point-occurrence data and sets of electronic maps summarizing environmental/ecologic dimensions that may or may not be important in limiting species' geographic distributions. These associations constitute an approximation of species' fundamental ecologic niches (the conjunction of ecologic conditions in which a species is able to maintain populations without immigration) (4) and hence provide a basis for understanding numerous ecologic and geographic phenomena related to species distributions.

We applied ecologic niche modeling to identify host relationships of *Triatoma* species and subspecies implicated in the transmission of Chagas disease. Previous studies by Ryckman (1) provide an ideal test case: hypotheses of association developed based on the modeling approach can be tested independently by using associations identified in Ryckman's detailed field studies. If successful, this approach would be invaluable in identifying host relationships for species for which detailed information is not available, for stratifying Chagas disease risk areas, and for planning the operational aspects of vector control strategies.

Methods

Point-Occurrence Information

Distribution data for members of the protracta species group were obtained from multiple sources (5–11; state vector control programs in Morelos and San Luis Potosí, unpub. data). Distribution data for *Neotoma* woodrats occurring in mainland Mexico (excluding offshore islands) were drawn from the Atlas of the Mammals of Mexico (Comisión Nacional para el Conocimiento y Uso de la Biodiversidad, unpub. data) and Hall (12) (textual localities only, georeferenced by

*Natural History Museum, Lawrence, Kansas, USA; †Universidad Nacional Autónoma de México, D.F., México; ‡Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and §Centro de Investigaciones sobre Enfermedades Infecciosas (CISEI), Cuernavaca, Morelos, México

hand from 1:50,000-scale maps to approximately 1 km precision). All occurrence data were georeferenced to the nearest 0.001° and organized in Excel 2000 (Microsoft Corp., Redman, WA) spreadsheets for analysis.

Distribution Modeling

Ecologic niches and potential geographic distributions were modeled with the Genetic Algorithm for Rule-Set Prediction (GARP) (13–15), a complex computer application that provides a broader, more objective approach than traditional Geographic Information System (GIS)-based approaches (3) but which yields GIS coverages as output. In general, the procedure focuses on modeling ecologic niches (16). Specifically, GARP relates ecologic characteristics of known occurrence points to those of points randomly sampled from the rest of the study region and develops a series of decision rules that summarizes those factors associated with the species' presence (3).

All modeling in this study was carried out on a desktop implementation of GARP in a beta-testing stage (R. Scachetti-Pereira, unpub. data). In this software package, occurrence points are divided evenly into training and test data sets. The GARP program works in an iterative process of rule selection, evaluation, testing, and incorporation or rejection: a method is chosen at random from a set of possibilities (e.g., logistic regression, bioclimatic rules), applied to the training data, and a rule is developed ("evolved," in the terminology of genetic algorithms). At each iteration in the program's processing, predictive accuracy is then evaluated based on 1,250 points resampled from the test data and 1,250 points randomly sampled from the study region as a whole. Rules may evolve in several ways that genetic algorithms use to mimic DNA evolution (e.g., point mutations, deletions, crossing over). The program uses the change in predictive accuracy from one iteration to the next to evaluate whether a particular rule should be incorporated into the model; the algorithm runs 1,000 iterations or until convergence.

The desktop GARP implementation offers much-improved flexibility (17) in choice of predictive environmental/ecologic GIS data coverage. In this case, we used 11 data layers summarizing elevation; slope; aspect (from the U.S. Geological Survey's HYDRO 1K data set, http://edcdaac.usgs.gov/gtopo30/hydro/); and aspects of climate, including cloud cover; daily temperature range; mean annual precipitation; maximum, minimum, and mean annual temperatures; vapor pressure; and wind speed (annual means 1960–1990; from the Intergovernmental Panel on Climate Change, http://www.ipcc.ch/). Analysis was limited to Mexico, because of the availability of distribution data for *Triatoma*, and cell resolution was set at 1x1 km. GARP's predictive abilities have been tested and proven under diverse circumstances (3,17–23).

To optimize model performance, we developed 100 replicate models of each species' ecologic niche based on random 50-50 splits of available occurrence points. Unlike previous applications, which either used single models to predict species' distributions (20) or summed multiple models to incorpo-

rate model-to-model variation (19), we used a new procedure (Peterson et al., unpub. data) for choosing best subsets of models. The procedure is based on the observations that 1) models vary in quality, 2) variation in models involves an inverse relationship between errors of omission (leaving out true distribution area) and commission (including areas not actually inhabited), and 3) best models (as judged by experts blinded to error statistics) are clustered in a region of minimum omission of independent test points and moderate area predicted (an axis related directly to commission error). The relative position of the cloud of points relative to the two error axes provides an assessment of the relative accuracy of each model. To choose best subsets of models, we eliminated all models that had nonzero omission error based on independent test points, calculated the average area predicted present in these zero-omission points, and identified models that were within 1% of the overall average. For species or subspecies for which fewer than 10 distribution points were available (which would have a weak extrinsic test of model quality) we developed 20 replicate models based on all points available and summed them as a "best" distribution hypothesis. Five species or subspecies were omitted because of the small sample size (e.g., *T. incrassata*, with one locality known in Mexico) or distribution outside Mexico (e.g., *T. protracta navajoensis*), leaving six taxa for analysis: *T. p. protracta*, *T. p. woodi*, *T. p. zacatecensis*, *T. peninsularis*, *T. sinaloensis*, and *T. barberi*.

Projection of the rule-sets for these models onto maps of North America provided distribution predictions. Model quality was tested by the independent sets of points (50%) set aside before GARP modeling: a chi-square test was used to compare observed success in predicting the distribution of test points with that expected under a random model (proportion of area predicted present x number of test points = expected predictive success if points and predictions were random with respect to each other).

Host-Ectoparasite Relationships

The protracta species complex is unusual in that Ryckman studied and documented the ectoparasite-host relationships (1), which provided an independent source of information regarding *Triatoma*-rodent interactions. In ArcView (version 3.2, ESRI, Redlands, CA), we calculated areas shared between each species or subspecies of the protracta species complex and each species of *Neotoma* woodrat in Mexico, as well as total modeled distribution areas for each species. We calculated the percent of distribution area (at the highest predicted level in the summed GARP outputs) that each *Triatoma* species or subspecies shares with each *Neotoma* species and assumed the most complete overlap values as suggesting an interaction between the species. We then tested these predictions by using the independent information provided by Ryckman (1), asking if species pairs with highest overlap values coincided with interactions identified in Ryckman's detailed field studies.

Results

Predictions of distributions for all species and subspecies for which ≥10 points were available were highly statistically significant (all p<0.001) and indeed coincided well with our understanding of known distributions of both insects (JMR and CBB) and woodrats (VSC). For example, *T. barberi* was predicted to extend broadly across central and southern Mexico, and populations were predicted in several regions (e.g., northern and eastern Michoacán and southern Hidalgo) for which previous occurrence data were not available. After analysis, this species was collected at Tlamaya, Hidalgo (J.C. Noguez-García, pers. comm.), and in northeastern Michoacán (E. Navarro, pers. comm.). Based on the random 50% resampling, in which half the data are set aside to provide an independent test of model quality, the 12 best-subset GARP models for this species were highly statistically significant (average p<10^{-114}). The overprediction of the distribution area for this species in Chiapas represents prediction into areas not inhabited for historical reasons, such as speciation, extinction, or limited dispersal ability (18). Tests for *Neotoma* species (Figure 1) were also highly significant (*N. albigula* p<10^{-18}, *N.*

goldmani p<10^{-16}, *N. lepida* p<10^{-11}, *N. mexicana* p<10^{-5}, *N. micropus* p<10^{-13}, and *N. phenax* p<10^{-73}); some areas of overprediction (e.g., *N. mexicana* in the Yucatan Peninsula) represent either prediction into areas not inhabited for historic reasons (18) or true overprediction error. Although sufficient point-occurrence data were not available for all species (Figure 2) to permit parallel tests, we are confident in the predictability of our distribution models.

Overlap of areas in *Triatoma* and *Neotoma* species and subspecies varied considerably (Table). For example, of the total modeled distribution area of *T. peninsularis*, 93.8% of the highest confidence prediction coincided with the distribution of *N. lepida* (Figure 3). Indeed, the overlap values were highly bimodal (Figure 4), suggesting that species' distributions either coincide or do not overlap, rather than the intermediate peak that might be expected if species did not interact.

The species pairs exhibiting maximum overlap values between *Triatoma* and *Neotoma* species (Table) coincided closely with species associations identified by Ryckman (1). Of the six *Triatoma* analyzed, the maximum overlap values coincided exactly with Ryckman's identified associations for four of the species. Of the other two species, *T. sinaloensis* shared 95.5% of its modeled geographic distribution with *N. phenax*, an interaction confirmed by Ryckman. However, our model showed no overlap with *N. albigula*; yet Ryckman found that these two species interacted, the complication is that sample sizes for *T. sinaloensis* were so small (n = 4) that its distribution was underpredicted. Relaxing the criterion for overlap to include areas predicted present by any of the best-subsets models showed an overlap of 33.2% with *N. albigula*. The final *Triatoma* species analyzed, *T. barberi*, was found by Ryckman (1) exclusively around human domiciles; nevertheless, its geographic distribution coincided closely with that of *N. mexicana* (98.4% overlap).

Discussion

Ecologic niche modeling and distribution prediction with GARP provides a powerful new tool for applications to dis-

Figure 1. Modeled geographic distributions of *Neotoma* woodrats in mainland Mexico. (A) black = *Neotoma fuscipes*, red = *N. phenax*, green = *N. mexicana* (note that this distribution includes those of other, less widely distributed, species), yellow = *N. goldmani*, blue = *N. palatina*, orange = *N. angustapalata*. (B) red = *N. albigula*, yellow = *N. micropus*, green = *N. albigula* and *N. micropus*.
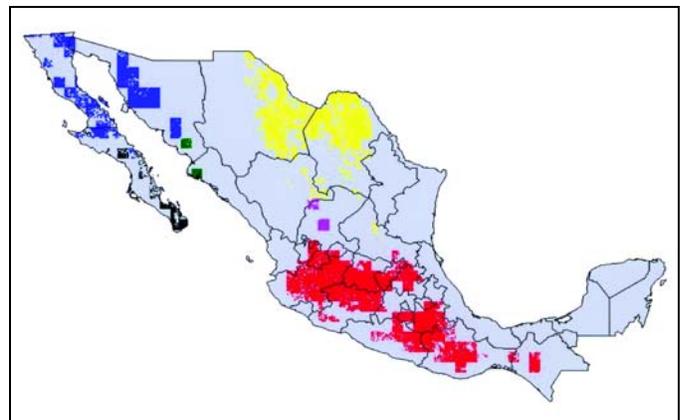
Figure 2. Modeled geographic distributions of members of the protracta species complex: red = *Triatoma barberi*, yellow = *T. p. woodi*, green = *T. sinaloensis*, blue = *T. p. protracta*, black = *T. peninsularis*, and pink = *T. p. zacatecensis*. Only areas predicted for each species at the highest level of confidence (all best-subsets models agree) are shown.

Table. Distribution of the six species of the protracta complex and nine species of *Neotoma* found in mainland Mexico[a]

	Distribution % of triatomine overlapping *Neotoma*									
	N. phenax	*N. palatina*	*N. micropus*	*N. mexicana*	*N. lepida*	*N. goldmani*	*N. fuscipes*	*N. angustapalata*	*N. albigula*	n
Triatoma barberi	0.0	2.5	45.1	98.4	0.0	0.1	0.0	0.0	30.0	86
T. sinaloensis	**95.5**	0.0	50.8	51.5	0.0	0.0	0.0	0.0	**0.0**	9
T. peninsularis	0.0	0.0	0.0	9.0	**93.8**	0.0	82.0	0.0	1.7	9
T. p. woodi	0.0	0.0	**94.6**	50.0	0.0	33.3	0.0	0.0	**96.1**	7
T. p. protracta	4.4	0.0	6.9	9.2	**24.4**	0.0	**39.3**	0.0	**14.1**	13
T. p. zacatecensis	0.0	0.0	100.0	100.0	0.0	24.3	0.0	0.0	**100.0**	9
Points available	31	5	69	103	16	13	4	5	156	

[a]Shown in bold are *Neotoma* species independently identified as hosts for particular *Triatoma* species (1). Distributions are to the highest confidence interval.

ease vectors and reservoirs. Even in systems more poorly understood than that examined here, patterns of overlap in geographic or ecologic space can provide initial hypotheses of host associations and disease reservoir or vector species. In this case, we were able to develop rigorous distribution hypotheses for 15 species of mammals and insects that interact in the potential transmission of Chagas disease in Mexico. These distribution hypotheses can form the basis for many applications in this field, including simple distribution prediction (for example, 3,17,20–22), analysis of specific parameters of species' ecologic niches (20), prediction of species' distributions across scenarios of climate change (24,25), prediction of species' invasions (19), assessment of patterns of evolutionary change in ecologic parameters (18; Martinez-Meyer et al., unpub. data), and spatial/epidemiologic stratification of disease endemic areas.

In general, the species pairs identified by ecologic niche modeling and evaluation of overlap of predicted geographic distributions coincided exactly with the interacting species
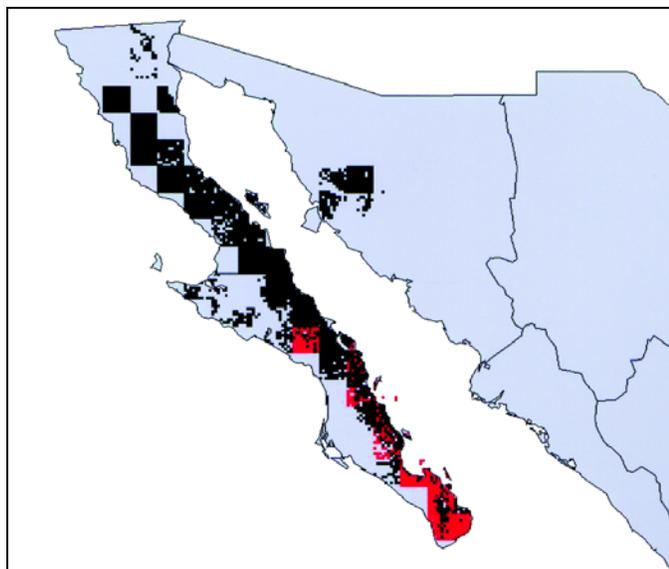
Figure 3. Modeled geographic distributions of *Triatoma peninsularis* (red) and *Neotoma lepida* (black), showing the tight geographic correspondence between the distribution of insect and host mammal. Almost all (93.8%) of the distribution area of *T. peninsularis* overlaps the distribution area of *N. lepida*.

pairs identified in detailed field studies by Ryckman (1). This result suggests that interactions between *Triatoma* and *Neotoma* have implications at a geographic level. That is, a *Triatoma* species does not simply infest the nests of whichever *Neotoma* are present at a particular site; rather, geographic distributions of *Triatoma* species tend to conform closely to those of their *Neotoma* hosts, suggesting a longer term evolutionary relationship. More detailed ecologic and behavioral studies focused on *Triatoma-Neotoma* interactions would be invaluable in clarifying the basis for this geographic-scale distribution coincidence.

The only exception to tight coincidence between modeled distribution overlap and Ryckman's identifications of species interactions was that of *T. sinaloensis* with *N. albigula*. This failure is clearly related to the minimal sample size on which the model for *T. sinaloensis* was based. With increased sample sizes, clearer identification of this interaction should be feasible. Other cases, such as the high overlap of *T. p. zacatecensis* with *Neotoma mexicana* and *N. micropus* (besides the high overlap with its host *N. albigula*), merit close examination in on-site field studies.

The most interesting case is that of *T. barberi*. Ryckman (1) identified this species as solely associated with human domiciles and did not find it associated with any rodent species. In Oaxaca, this species has been collected in sylvatic habitats near rock outcroppings, but specific host species have not
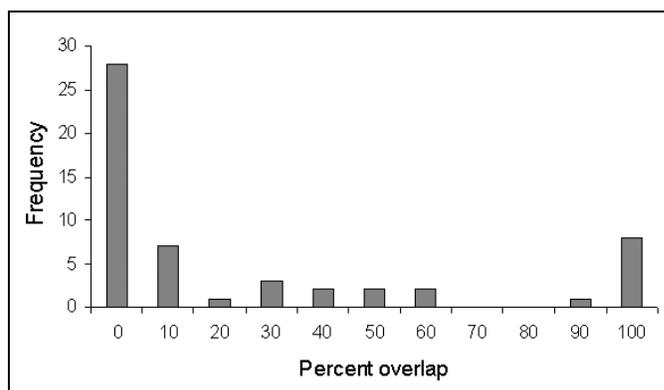
Figure 4. Frequency distribution of areas of overlap between *Triatoma* and *Neotoma* species, expressed as percent of total modeled area (at highest predictive level) of the *Triatoma*.
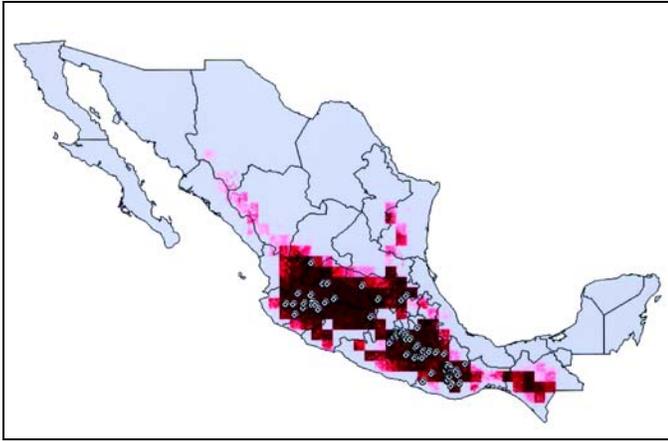
Figure 5. Modeled geographic distribution of *Triatoma barberi*, shown with known occurrence points used to create and test the ecologic niche model. Dark red = 100% of best-subsets models predict presence, medium red = 75% of best-subsets models predict presence, light red = 50% of best-subsets models predict presence, lightest red = any best-subsets model predicts presence.

been identified (7). The species' extensive geographic distribution (Figure 5) coincided closely (98.4% overlap) with that of *N. mexicana*, at a level that for other *Triatoma* species would almost certainly indicate an interaction. We suggest two possibilities, which are not mutually exclusive: 1) that *T. barberi* does indeed parasitize *N. mexicana* nests, but that additional sampling is necessary to detect this association, or 2) that *T. barberi* may originally have been an ectoparasite of *N. mexicana*, but from that host it made the transition to a domestic or peridomestic existence.

Species' distribution predictions and our capacity to link these distributions with disease transmission are novel epidemiologic tools. Understanding sylvatic transmission cycles and invasion of peridomestic habitats is an immediate application for niche analysis and modeling. This general approach has potential applications much broader than the protracta species complex and *Neotoma* associations as they relate to Chagas disease transmission. We anticipate addressing host relationships in other *Triatoma* such as the phyllosoma group, the most important complex of vector species of Chagas disease in Mexico and for which host relationships are all but unknown (7). Sylvatic reservoir species for many tropical diseases remain poorly documented (e.g., leishmaniasis, hantavirus pulmonary syndrome, West Nile encephalitis, leptospirosis) and our approach provides a strategy for narrowing the field of possible species and ecologic scenarios.

Dr. Peterson is associate professor of ecology and evolutionary biology and curator of ornithology in the Natural History Museum and Biodiversity Research Center, University of Kansas. His research interests focus on understanding factors affecting species' geographic distributions, including aspects of evolution, phylogenetics, and geography.

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Address for correspondence: A. Townsend Peterson, Natural History Museum, University of Kansas, Lawrence, Kansas 66045, USA; fax: 785-864-5335; e-mail: town@ku.edu

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Ehrlichia ewingii Infection in White-Tailed Deer (*Odocoileus virginianus*)

Michael J. Yabsley,* Andrea S. Varela,* Cynthia M. Tate,* Vivien G. Dugan,*
David E. Stallknecht,* Susan E. Little,* and William R. Davidson*

Two closely related zoonotic ehrlichiae, *Ehrlichia chaffeensis* and *E. ewingii*, are transmitted by *Amblyomma americanum*, the lone star tick. Because white-tailed deer (*Odocoileus virginianus*) are critical hosts for all mobile stages of *A. americanum* and are important vertebrate reservoirs of *E. chaffeensis*, we investigated whether deer may be infected with *E. ewingii*, a cause of granulocytotropic ehrlichiosis in humans and dogs. To test for *E. ewingii* infection, we used polymerase chain reaction and inoculation of fawns with whole blood from wild deer. Of 110 deer tested from 20 locations in 8 U.S. states, 6 (5.5%) were positive for *E. ewingii*. In addition, natural *E. ewingii* infection was confirmed through infection of captive fawns. These findings expand the geographic distribution of *E. ewingii*, along with risk for human infection, to include areas of Kentucky, Georgia, and South Carolina. These data suggest that white-tailed deer may be an important reservoir for *E. ewingii*.

*E*hrlichia ewingii, one of the causative agents of canine granulocytotropic ehrlichiosis, has been reported in dogs in several U.S. states, including Oklahoma, North Carolina, and Virginia (1–4). Human infections with *E. ewingii* have been reported from Missouri, Oklahoma, and Tennessee (5,6); the clinical disease, similar to that caused by other *Ehrlichia* spp., is characterized by fever, headache, and thrombocytopenia, with or without leukopenia (5–7). Experimentally, the lone star tick (*Amblyomma americanum*) has been shown to be a competent vector (8); however, natural infection of two other tick species, *Rhipicephalus sanguineus* and *Dermacentor variabilis*, has been reported in Oklahoma (2).

The white-tailed deer (*Odocoileus virginianus*) is an important host for all three mobile stages of *A. americanum*, and deer and lone star ticks serve as the major reservoir and vector, respectively, for *E. chaffeensis* (9–11). Because *E. ewingii* is closely related to *E. chaffeensis* and shares the same vector, our goal was to determine if white-tailed deer are naturally infected with *E. ewingii*. In some human and canine infections with *E. ewingii*, cross-reactions with *E. chaffeensis* antigens have been reported (5,6); however, not all infections with *E. ewingii* result in positive serologic tests to *E. chaffeensis* antigen (2,6). Because *E. ewingii* has not been isolated in culture and because serologic test reagents are not readily available, we used several techniques to detect infections, including 1) testing serum samples for antibodies reactive with *E. chaffeensis* antigen, 2) testing leukocytes or whole blood by polymerase chain reaction (PCR) with primers specific for *E. ewingii* and *E. chaffeensis*, and 3) injecting captive white-tailed fawns with whole blood from deer collected in an *A. americanum*–endemic area.

Methods

From September 1996 to July 2001, whole blood samples and serum from 110 deer from 20 sites (Table 1) in the southeastern United States were collected in vacutainer EDTA tubes (whole blood) and serum tubes (Becton, Dickinson and Company, Franklin Lakes, NJ). For PCR, two blood preparation protocols were followed. During the 1996–1997 collection period, leukocytes were separated from whole blood as described (9); during the 2000–2001 period, whole blood was extracted for PCR assays. Both leukocytes and whole blood samples were frozen at –20°C until PCR testing was done. Serum samples were held in vials at –20°C until serologic testing.

Because *A. americanum* is the only experimentally proven vector for *E. ewingii*, locations with deer infested with *A. americanum* were selected for this study. Serum from each deer was tested for antibodies reactive to *E. chaffeensis* by the indirect immunofluorescent antibody (IFA) test as described (10), with the following modifications. Briefly, sera were screened at a dilution of 1:128 by using *E. chaffeensis* antigen slides obtained from Focus Technologies (formerly MRL Diagnostics, Cypress, CA). A 1:50 dilution of fluorescein isothiocyanate-labeled rabbit anti-deer immunoglobulin G (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was used as conjugate.

DNA from 200 µL whole blood or 20 µL leukocytes was extracted by using the GFX Genomic Blood DNA Purification Kit (Amersham Biosciences, Piscataway, NJ) and InstaGene Purification Matrix (Bio-Rad Laboratories, Hercules, CA), respectively, following the manufacturer's protocol. Primary outside amplification consisted of 5 µL DNA from whole blood or 10 µL from leukocytes in a 25-µL reaction containing 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (dNTP), and 2.5 units Taq

*University of Georgia, Athens, Georgia, USA

Table 1. Polymerase chain reaction (PCR) results for *Ehrlichia chaffeensis* and *E. ewingii* in 110 white-tailed deer, southeastern United States

Location[a]	County/state	*E. chaffeensis* PCR no. positive/ no. tested (%)	*E. ewingii* PCR no. positive/ no. tested (%)
White River NWR	Arkansas, AR	0/5	0/5
Felsenthal NWR	Ashley, AR	0/5	0/5
Pea Ridge NMP	Benton, AR	1/6 (17)	1/6 (17)
Shirey Bay WMA	Lawrence, AR	0/5	0/5
Cache River NWR	Monroe, AR	1/5 (20)	0/5
St. Vincent NWR	Franklin, FL	0/4	0/4
White Oak CC	Nassau, FL	0/5	0/5
Piedmont NWR	Jones, GA	0/5[b]	0/5[c]
St. Catherines Island	Liberty, GA	0/5	0/5
Blackbeard Island	McIntosh, GA	1/7 (14)	2/7 (29)
Harris Neck NWR	McIntosh, GA	0/5	0/5
Ballard WMA	Ballard, KY	0/5	0/5
Fort Knox	Hardin, KY	0/5	0/5
West Kentucky WMA	McCracken, KY	1/5 (20)	1/5 (20)
Tensas River NWR	Madison, LA	0/3	0/3
Dahomey NWR	Bolivar, MS	0/3	0/3
Cape Hatteras NS	Dare, NC	1/4 (25)	1/4 (25)
Mattamukseet NWR	Hyde, NC	1/5 (20)	0/5
Sea Pines	Beaufort, SC	0/18	1/18 (6)
Kiawah Island	Charleston, SC	0/5	0/5
Total		6/110 (5.5)	6/110 (5.5)

[a]NWR, National Wildlife Refuge; NMP, National Military Park; WMA, Wildlife Management Area; CC, Conservation Center; NS, National Seashore.
[b]At least 1 (20%) of 5 was positive based on transmission to fawn 81.
[c]At least 2 (40%) of 5 were positive based on transmission to both fawns 76 and 81.

DNA Polymerase (Promega Corp., Madison, WI), and 0.8 µM of primers ECC and ECB (11). For the nested PCR, 1 µL of primary product was used as template in a 25-µL reaction containing the same PCR components, except for the addition of *E. ewingii*-specific primers, EE72-ewingii (5'-CAATTC-CTAAATAGTCTCTGACTATT-3') and HE3 (4), or *E. chaffeensis*-specific primers, HE1 and HE3 (11). Amplified products were separated in 2% agarose gels, stained with ethidium bromide, and visualized with UV light. Representative secondary PCR products for *E. ewingii* were purified with a Microcon spin filter (Amicon Inc., Beverley, MA), sequenced with an ABI 3100 automated sequencer (Applied Biosystems, Perkin Elmer Corp, Foster City, CA), and then compared with published *E. ewingii* sequences (GenBank accession nos. M73227 [3] and U96436 [1]).

Two 4-month-old, laboratory-reared white-tailed fawns (76 and 81) were housed in a tick-free facility. Before inoculation both fawns were negative for antibodies reactive to *E. chaffeensis* and PCR-negative for both *E. chaffeensis* and *E. ewingii*. Whole blood for injection was obtained from five wild source deer (WTD 1–5) collected at Piedmont National Wildlife Refuge (NWR) in Jones County, Georgia, on July 24, 2001. A whole blood sample from each wild deer was also cultured in DH82 canine macrophage cells as described (12).

Fawns were anesthetized by intramuscular injection of tiletamine HCL and zolazepam HCL (4.4 mg/kg body weight; Fort Dodge Animal Health, Fort Dodge, IA) and xylaxine (2.2 mg/kg; Butler, Columbus, OH) and were reversed with intravenous injection of yohimbine (0.125 mg/kg; Lloyd Laboratories, Inc., Shenandoah, IA). Equal volumes of whole blood in EDTA from WTD1–3 were pooled, and a total of 8 mL was injected into fawn 76 in 2-mL aliquots by each of four routes (intravenous, intradermal, subcutaneous, and intraperitoneal). Fawn 81 was injected in the same way with a total of 8 mL of pooled blood from WTD4 and WTD5. Blood samples were collected from both fawns on 5, 9, 15, 20, 47, 68, and 110 days postinjection (DPI) for PCR, serologic tests, and blood smears. Blood was tested by PCR for *E. ewingii* and *E. chaffeensis* as described above and for the human granulocytotropic ehrlichiosis (HGE) agent (*Anaplasma phagocytophila*) by using primers GE9f and GA1UR, as described (13).

Results

Ninety-seven (88.1%) of the 110 wild deer had antibodies reactive (≥1:128 titer) to *E. chaffeensis* by IFA testing. All locations examined contained seropositive deer (range 57%–100%). A 407-bp product characteristic of *E. ewingii* was generated in six (5.5%) deer by nested PCR, and six (5.5%) deer were also positive for *E. chaffeensis* (Table 1). Positive PCR results for *E. chaffeensis* and *E. ewingii* were obtained with both blood preparation processes. Only one deer (0.9%) was positive for both *E. ewingii* and *E. chaffeensis* by PCR.

All five source deer (WTD1–5) were positive for antibodies to *E. chaffeensis,* but negative by PCR for *E. ewingii* and *E. chaffeensis* (Table 2). However, blood from deer WTD5 was culture positive for *E. chaffeensis*. Fawn 81 was at first positive for antibodies reactive to *E. chaffeensis* at 15 DPI, tested negative at 20 DPI, and was positive at 47, 68, and 110 DPI. Fawn 76 was seronegative on all days tested. Both fawns were PCR positive for *E. ewingii* at 47 DPI, and fawn 81 remained PCR positive at 68 DPI (Table 2). Whole blood samples from

Table 2. Summary serologic and polymerase chain reaction (PCR) data for fawns injected with pooled blood from infected source white-tailed deer[a] (WTD1–5)

Fawns	IFA results	*E. ewingii* (DPI)[b]	*E. chaffeensis* (DPI)	HGE
		PCR results		
Fawn 76 (received blood from WTD1–3)	—	+ (47)	—	—
Fawn 81 (received blood from WTD 4–5)	+	+ (47, 68)	+ (15, 20, 47, 68, 110)	—

[a]The five source deer (WTD 1–5) were positive by indirect immunofluorescent antibody (IFA) test (titer ≥128) and negative by PCR for *Ehrlichia ewingii, E. chaffeensis*, and the HGE agent (*Anaplasma phagocytophila*).
[b]DPI, days post inoculation; HGE, human granulocytotropic ehrlichiosis.

fawn 81 were PCR positive for *E. chaffeensis* at 15, 20, 47, 68, and 110 DPI. On thin blood smears taken at 47 DPI, morulae characteristic of *E. ewingii* were observed in approximately 2%–3% and <1% of neutrophils of fawns 81 and 76, respectively (Figure). Both deer remained PCR negative for the HGE agent.

Sequences of three *E. ewingii* products (Dare County, North Carolina; Fawn 76; and Fawn 81) were identical to published gene sequences M73227 and U96436. The *E. ewingii* product from Benton County, Arkansas, differed from the others at base 225, which corresponds to GenBank accession number AY093439. The *E. ewingii* sequences were deposited in the GenBank database under accession numbers AY093439–AY093441 and AY497628.

Discussion

Our data provide the first evidence that white-tailed deer are naturally infected with *E. ewingii*; this information extends the geographic distribution of *E. ewingii* to include areas of Kentucky, Georgia, and South Carolina. Before this report, the only reported vertebrate hosts for *E. ewingii* were humans and dogs. By combining data from PCR and injection studies, we showed that at least 8 (7.3%) of 110 deer were infected with *E. ewingii*, which is similar to prevalence rates previously reported for dogs. Infection with *E. ewingii* has been reported in 6.2%–15.8% of dogs from southeastern Virginia, Oklahoma, and southeastern North Carolina (2,4,14). Because of the unknown sensitivity of PCR for detection of this organism, this percentage may represent a substantial underestimation of
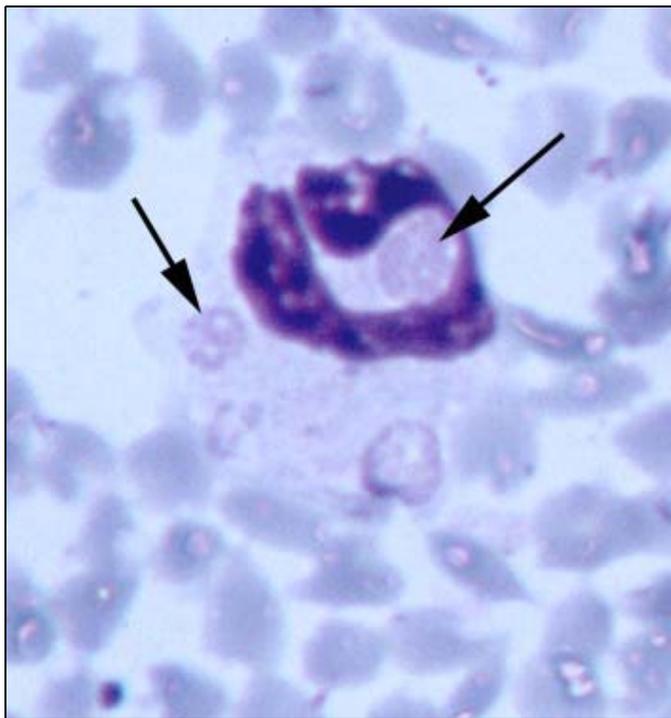
Figure. Multiple morulae consistent with *Ehrlichia ewingii* in a neutrophil from fawn 81 experimentally injected with pooled whole blood from two wild white-tailed deer (Giemsa stain, 100X).

the actual prevalence of *E. ewingii* infection in white-tailed deer. Our data suggest that the distribution of *E. ewingii* and hence the risk for human and canine infection may be more widespread than previously reported and may correspond with the distribution of *A. americanum*.

Although whole blood samples from all five deer (WTD1–5) collected at Piedmont NWR in Georgia were negative by PCR, *Ehrlichia* spp. infections developed in both inoculated fawns. Therefore, at least two of the Piedmont NWR deer were infected with *E. ewingii*, since *E. ewingii* infection was identified in both fawns. In addition, at least one Piedmont NWR deer was positive for *E. chaffeensis*, as fawn 81 became infected and WTD5 was culture positive. Because a much smaller volume of blood was used for PCR (20–200 μL) than for culture (5 mL) or injection of fawns (8 mL), low numbers of organisms may have been more readily detected by the other two methods. Consistent with results of previous studies (12,15), our data indicate that use of PCR alone as a screening tool may fail to detect acute infections of white-tailed deer with *Ehrlichia* spp.

Although fawn 76 was clearly infected with *E. ewingii* on the basis of PCR and detection of morulae, its results were never positive by serology. Serologic cross-reactions between *E. ewingii* and *E. chaffeensis* have been reported (5,6); however, not all *E. ewingii*-infected dogs or humans develop antibodies to *E. chaffeensis* antigens (2,6). Compared with previous experimental infections of white-tailed deer with *E. chaffeensis* (11,15), an extended period of time was required before *E. ewingii* was detected. Low numbers of *E. ewingii* in the original inoculum may explain the longer time required for PCR detection of *E. ewingii* in fawns 76 and 81. Because this experimental infection was a small pilot study, limited insight is provided into the course of *E. ewingii* infection in white-tailed deer. However, the detection of *E. ewingii* in fawn 81 over a 3-week period indicates that *E. ewingii* was capable of replicating in white-tailed deer.

White-tailed deer have been demonstrated as important reservoirs for *E. chaffeensis* (11,12,15). In this study, using PCR, culture, and inoculation of fawns, at least 7 (6.4%) of 110 deer were positive for *E. chaffeensis*. In previous studies in *A. americanum*–endemic areas, as many as 40%–100% of white-tailed deer have been shown to have antibodies reactive with *E. chaffeensis*, and up to 20% of deer are PCR positive (10,12). Five of the seven populations of white-tailed deer positive for *E. chaffeensis* were also positive for *E. ewingii*. This finding is not surprising, as these pathogens share the same vector. Although evidence of the HGE agent has been detected in white-tailed deer by both serologic testing and PCR (13,16), the relative importance of deer as reservoirs for the HGE agent has not been fully evaluated. Although our study demonstrates that white-tailed deer can harbor a third human ehrlichial pathogen, the importance of deer as a reservoir is not known.

Data from this study raise several important issues: 1) because of epidemiologic similarities between *E. chaffeensis* and *E. ewingii*, deer could be an important reservoir for *E.*

ewingii; 2) because of potential serologic cross-reactivity, *E. chaffeensis* seroreactors in the current and prior surveys of white-tailed deer (10,17) could actually represent *E. chaffeensis*, *E. ewingii,* or mixed infections; and 3) because at least four *Ehrlichia* species infect white-tailed deer (*E. chaffeensis*, *E. ewingii*, *A. phagocytophila*, and an undescribed *Ehrlichia* sp.) (9,12,13,16), an array of diagnostic assays should be used for detecting *Ehrlichia* spp. infections. Therefore, further studies are needed to examine the reservoir potential of white-tailed deer for *E. ewingii* and other ehrlichal infections.

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Mr. Yabsley is a doctoral student in the College of Veterinary Medicine at the University of Georgia. His area of research is the epidemiology of zoonotic parasites, with a particular focus on tick-borne pathogens.

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Address for correspondence: Michael J. Yabsley, Wildlife Health Building, Southeastern Cooperative Wildlife Disease Study, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA; fax: 706-542-5865; e-mail: myabsley@vet.uga.edu

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Time-Space Clustering of Human Brucellosis, California, 1973–1992[1]

Geoffrey T. Fosgate,* Tim E. Carpenter,* Bruno B. Chomel,* James T. Case,†
Emilio E. DeBess,‡ and Kevin F. Reilly§

Infection with *Brucella* spp. continues to pose a human health risk in California despite great strides in eradicating the disease from domestic animals. Clustering of human cases in time and space has important public health implications for understanding risk factors and sources of infection. Temporal-spatial clustering of human brucellosis in California for the 20-year period 1973–1992 was evaluated by the Ederer-Myers-Mantel, Moran's I, and population-adjusted Moran's I procedures. Cases were clustered in concentrated agricultural regions in the first 5-year interval (1973–1977). Time-space clustering of human brucellosis cases in California late in the 20-year study period may reflect the distribution of Hispanic populations. Public health programs in California should focus on educating Hispanic populations about the risk of consuming dairy products, such as soft cheeses, made from unpasteurized milk.

Brucellosis is associated with chronic debilitating infections in humans and reproductive failure in domestic animals (1–3). Person-to-person transmission of brucellae is extremely rare (2,4), and human infection may be an accidental expression of a more widespread problem in animals (5). *Brucella* species considered important agents of human disease are *B. abortus* (primary reservoir in cattle), *B. melitensis* (sheep and goats), and *B. suis* (swine) (6). *B. melitensis* and *B. suis* are considered more pathogenic for humans than *B. abortus* (7). Dogs are reservoirs of *B. canis*; human infection has been documented to result in disease (3,8,9) but does not constitute an important public health concern in the United States (2,10).

Control and eradication of brucellosis in domestic animals have important public health implications. Test-and-slaughter programs, in conjunction with vaccination, are the major method of control (11). Whole herd depopulation (12) can also be used when other methods have reduced disease prevalence to low levels. Livestock populations can be screened for brucellosis by serologic testing of individual animals (12–16) or by testing pooled samples such as bulk milk (12,14). Several vaccines are available for reducing infection in animal populations (11), thereby reducing transmission potential to humans.

Persons infected with *Brucella* spp. usually have signs and symptoms consistent with an influenzalike or septicemic illness, often with insidious onset. The symptoms and clinical signs most commonly reported are fever, fatigue, malaise, chills, sweats, headaches, myalgia, arthralgia, and weight loss (8,10,17,18). Fewer than 10% of human cases of brucellosis may be recognized and reported (19), likely because of this misleading clinical picture (2,8). The acute form of human brucellosis is characterized by an undulating fever, in addition to the signs and symptoms mentioned. Lack of appropriate therapy during the acute phase may result in localization of bacteria in various tissues and lead to subacute or chronic disease that can have serious clinical manifestations (6,8,10).

Most cases involving field strains of *Brucella* can be traced to domestic food animals (5), and the prevalence of disease in livestock reservoirs reflects its occurrence in humans. Commonly, *B. abortus* and *B. suis* infections are associated with certain occupational groups, including farm workers, veterinarians, and meatpacking employees (6). Human *B. melitensis* infections occur more frequently in persons who do not have these occupational exposures (10). Persons usually become infected with brucellae through direct contact with infected animals or their products. Unpasteurized milk and processed dairy foods from infected animals are the major source of infection for the general population (7,10), and infected carcasses are the source of infection for workers in the meatpacking industry (20–22). Veterinarians can acquire brucellosis from assisting births in infected animals, as well as through inadvertent exposure to *B. abortus* strain 19 vaccine (5). Airborne transmission of bacteria to humans has also been documented in clinical laboratories and abattoirs (21,23). Protective clothing and careful handling of infected animals can reduce occupation-related brucellosis (6,24), and avoiding unpasteurized dairy products should prevent infection in the general population (20).

The epidemiologic pattern of human infection with brucellae has been changing in the United States since 1947, when the number of reported cases was the highest ever recorded (6,321 total; 4.4 cases/100,000 population) (19,20). This change has been attributed to implementation of the state-federal Cooperative Brucellosis Eradication Program in 1934 and

*University of California, Davis, California, USA; †California Animal Health and Food Safety Laboratory, Davis, California, USA; ‡Department of Human Services, Portland, Oregon, USA; and §California Department of Health Services, Sacramento, California, USA

widespread milk pasteurization (19). *B. abortus* infection was common in the general population and before 1960 was the most frequent cause of human brucellosis (18). The relative importance of occupational exposures, especially in abattoir workers, steadily increased during the 1950s to 1970s (9,18,20,21). *B. suis* became the most frequent isolate in human cases from the mid-1960s to the early 1970s (9,18,19). The epidemiologic pattern of human brucellosis in the United States since the early 1970s may have shifted from an occupation-associated disease involving *B. suis* to one more common in the general population (23,25). This change may be attributed to the swine brucellosis eradication program implemented in 1961 (22) and increased reporting of human *B. melitensis* infection (8,23,25), which was considered to have been eradicated from U.S. sheep and goats in 1972 (4). Hispanic populations of California are at increased risk for *B. melitensis* infection, with imported soft cheese the most commonly reported vehicle of exposure (23,25–27).

The objective of our study was to evaluate time-space clustering of reported human brucellosis cases in California for the 20-year period from 1973 to 1992. Determination of high-risk zones for human brucellosis may be useful in focusing education programs and public health funding.

Materials and Methods

Human brucellosis is a reportable disease in California; data from 1973 to 1992 were obtained through the Office of Statistics and Surveillance and the Veterinary Public Health Section, California Department of Health Services. Data included county of residence, year of diagnosis, and patients' reported race and age. Data without personal identifiers were obtained for cases confirmed according to Centers for Disease Control and Prevention definitions (28). Basic descriptive data for reported cases of brucellosis from 1992 to 2001 were also obtained from the California Department of Health Services. California census information was downloaded from the Demographic Research Unit, California Department of Finance website, at http://www.dof.ca.gov/html/Demograp/druhpar.htm.

The 20-year study period was divided into four 5-year periods for calculating county-level human brucellosis incidence. The numerator for the incidence calculation was the total number of cases in residents of each county in the 5-year period. The denominator was the county population for the median year of the 5-year interval. Crude county-level incidences were calculated, as well as incidences adjusted for race and combined age and race. The data were adjusted for race by categorizing the total population into Hispanic and non-Hispanic segments and for age by grouping the population into <10-, 10–19-, 20–29-, 30–39-, 40–49-, 50–59-, and ≥60-year categories. Brucellosis incidence proportions were adjusted directly by using the population distribution of Sacramento County in 1990 as the standard. In brief, direct adjustment of proportions was done by first calculating the proportion of brucellosis in each specific age-race category for all counties.

This proportion was then multiplied by the number of persons in the corresponding age-race category of the standard population (Sacramento County, 1990) to yield the expected number of cases. This expected number was summed over all age-race categories for each county and then divided by the total standard population (total population of Sacramento County, 1990) to yield the adjusted proportion.

Because of its insidious onset, the actual date of onset of *Brucella* infection is often difficult to determine retrospectively. Therefore, the date of diagnosis for each case, rather than onset of symptoms, was used for calculating incidence. Cases were considered to have originated in the patient's county of residence for all calculations, although the person may not have become infected in that county. Data were not available to evaluate the effect of these assumptions on incidence calculations. ArcView Geographic Information System, version 3, (Redlands, CA) was used to visually represent spatial distribution of brucellosis cases and incidence proportions in California counties.

The Ederer-Myers-Mantel (EMM) procedure (29) was used to examine time-space clustering of human brucellosis cases in each California county during the 20-year study period. This one-sided test for clustering was implemented in an Excel spreadsheet program (Microsoft Corp., Redmond, WA). Separate race-specific analyses were also performed for cases in Hispanic and non-Hispanic residents. The EMM test is sensitive to departures from a static population over time and is therefore not recommended for situations involving more than five periods, as the baseline population may change. To account for this limitation, the 20-year period was divided into four 5-year periods in which the base population should not change meaningfully. This division of the period results in analysis of 232 counties (58×4) over 5 years each, instead of 58 counties over 20 years. Cases in Los Angeles County during 1978–1982, for instance, will not be linked to cases in Los Angeles before or after this 5-year period. Different periods for the same county are treated as completely independent.

The Moran's I test for spatial clustering (30) was performed to evaluate distribution of incidence proportions of human brucellosis in California counties during the study period. Data were analyzed by RAMAS Cast, version 2.0 (Applied Biomathematics, Sebauket, NY). This statistical procedure examines values in adjacent areas (counties), is two-sided, and calculates a standard normal z-score in which a positive test statistic indicates a tendency toward a clustered distribution and a negative statistic a tendency toward a uniform (dispersed) distribution.

The Moran's I test was performed independently for the four 5-year cumulative incidences of brucellosis in each California county during the study period. This analysis was also performed on the average incidence for each county during the entire 20-year period. Crude proportions as well as race- and age/race-adjusted proportions were analyzed.

A modification of the Moran's I technique, the population-adjusted Moran's I (I_{pop}), which adjusts for the underlying

population density in each area (31), was used to evaluate spatial clustering of reported human brucellosis cases in California. The procedure was performed by using RAMAS Cast (Applied Biomathematics). Similar to the unadjusted Moran's I, this statistical method is two-sided and calculates a standard normal z-score; however, the I_{pop} test is based on the numerator (number of cases) separate from the denominator (population at risk). Therefore, I_{pop} cannot be used for adjusted proportions because the data needed are numerators and denominators, rather than proportions.

The I_{pop} analysis was done by using numerator and denominator information from all four 5-year cumulative incidences of brucellosis in each California county during the study period. The total incidence for each county during the entire 20-year period was analyzed similarly, with mean county populations as the denominator. Spatial clustering was evaluated for the total population, as well as for Hispanic and non-Hispanic population segments.

Similar I_{pop} analyses were performed for reported cases in Hispanic and non-Hispanic segments of the population specific for *B. abortus* and *B. melitensis* infection. The causative *Brucella* species was determined either through bacteriologic isolation or determination of reported animal contact. Bacterial isolation was not done for all reported cases, and often the *Brucella* species was not determined because of concern about exposure risks for laboratory personnel. Patients reporting cattle as the primary animal contact were classified as having disease due to *B. abortus* when the infecting species was not determined. Similarly, patients reporting contact with goats (or goat cheeses) were classified as having infection with *B. melitensis*. This classification was necessary because the *Brucella* species involved was not identified for many cases.

Results

A total of 426 human cases of brucellosis were reported in California from 1973 to 1992. Ten cases were excluded from analysis because recorded permanent residence was not in California, leaving 416 for study. Except for the period 1974–1976, the number of Hispanic cases reported each year from 1973 to 1992 was greater than the total for all other ethnic groups combined (Figure 1). Hispanics accounted for 305
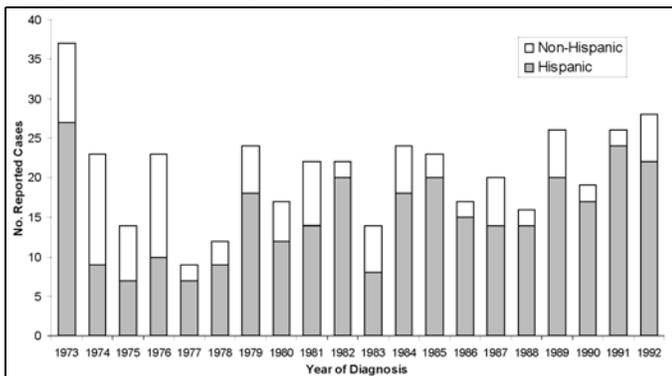
Figure 1. Number of reported cases of human brucellosis in Hispanic and non-Hispanic California residents, by year, 1973–1992.
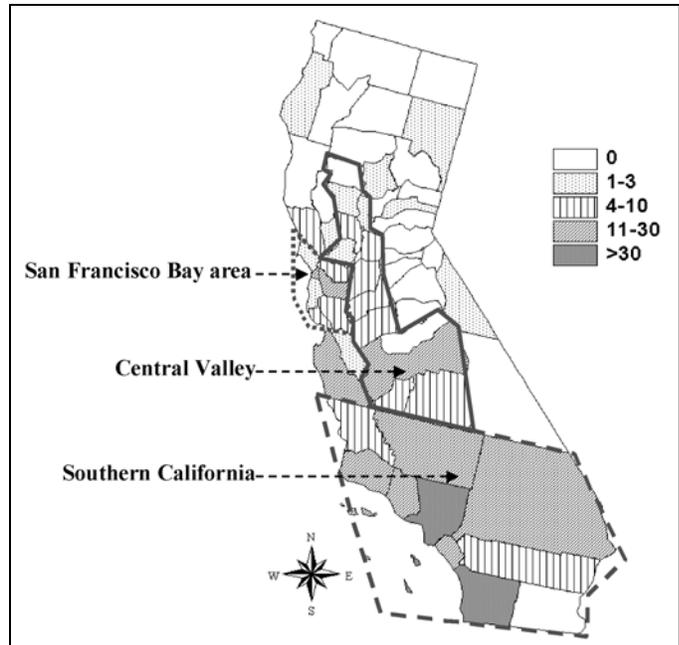
Figure 2. Distribution of reported cases of human brucellosis in California residents, 1973–1992.

(73%) of the reported cases in this 20-year period. The number of reported cases was highest in southern California (66%), with another 14% from the Central Valley and 12% from the San Francisco Bay area (Figure 2).

The *Brucella* species was identified in 231 (56%) of the 416 cases analyzed. *B. abortus* was isolated from 39 cases, *B. melitensis* from 181, *B. suis* from 9, and *B. neotomae* from 2. Expanding classification to include information about contact with animal species led to determination of 91 cases due to *B. abortus* and 200 cases due to *B. melitensis*.

Human brucellosis cases since 1992, reported mainly from southern California counties, also affected mostly Hispanics (Figure 3). Hispanics accounted for 185 (77%) of the 240 total cases reported in California from 1993 to 2001, which was similar to data for 1973–1992. Southern California counties accounted for 136 (57%) of the total cases; another 22% were from the Central Valley and 14% from the San Francisco Bay area. Counties reporting the largest number of cases were Los Angeles (53 cases), San Diego (30 cases), and Orange (19 cases).

According to the EMM procedure, clustering was statistically significant for Hispanic, non-Hispanic, and total cases (Table 1); this procedure was also used to determine relative contribution to overall clustering by individual county (Table 2). Most of the clustering effect during 1973–1982 in Hispanic cases was found in southern and Central Valley counties. Clustering was most pronounced in Los Angeles County, with 23 cases reported in 1973 although the maximum number of cases expected from the EMM procedure was 10 (chi square 74.9, p<0.001). During 1988–1992, substantial clustering of Hispanic cases also occurred in the San Francisco Bay area and southern California. Non-Hispanic cases were significantly
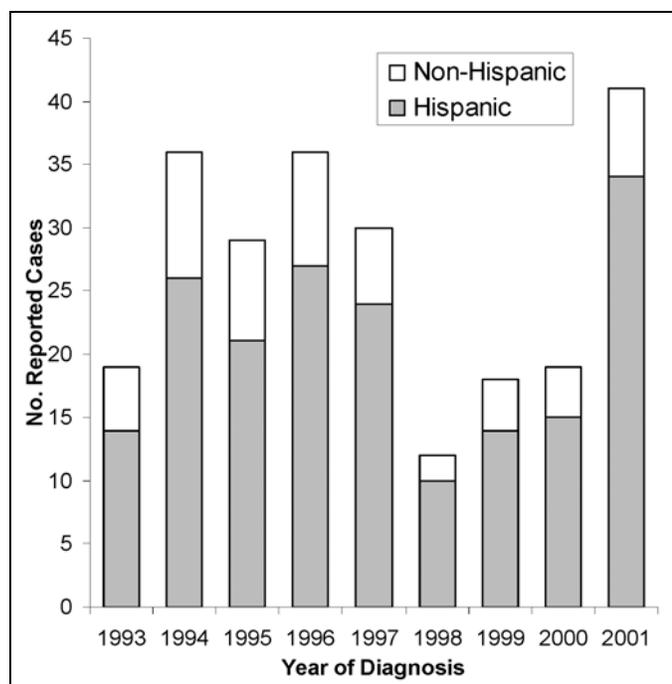
Figure 3. Reported cases of human brucellosis in Hispanic and non-Hispanic California residents, by year, 1993–2001.

clustered in the Central Valley and San Francisco Bay area during 1983–1987. Cases also clustered in southern California during 1988–1992.

The Moran's I procedure demonstrated significant spatial clustering of crude incidence of human brucellosis in California for the 5-year periods from 1973 to 1977 and 1983 to 1987 (Table 3). Spatial clustering remained statistically significant for 1973–1977 after incidences were adjusted for differences in age and race structure of the California counties, but significance during 1983–1987 was removed by this adjustment (Table 3). The largest adjusted incidences of human brucellosis occurred in the Central Valley, the most active agricultural region of California, during 1973–1977 (Figure 4). Spatial distribution of large adjusted incidences shifted away from highly agricultural zones in the later periods, and clustering was not statistically significant.

When the population as a whole was examined, the I_{pop} procedure showed substantial spatial clustering in all four 5-year study periods (Table 4). Significant clustering was also observed in the Hispanic-specific California population for all study periods except 1983–1987. Cases in the non-Hispanic population were not clustered during any period. The clustering effect in all tests resulted more from the number of cases within counties than from cases in adjacent counties (Table 4).

Significant spatial clustering was present in Hispanics infected with *B. abortus* during 1978–1982 (Table 5) and also in Hispanic populations infected with *B. melitensis* for all 5-year periods except 1983–1987. Cases of *B. abortus* or *B. melitensis* infection in non-Hispanic populations were not significantly clustered at any time during the 20-year study period.

Discussion

The Hispanic segment of the California population has been shown to be at a higher risk for brucellosis during the period from the early 1970s to the early 1990s (25). The number of cases reported during 1973–1992 was higher in Hispanics than in all other ethnic groups combined. Spatial distribution of cases was centered in southern California and other areas of the state with large Hispanic populations.

Results of the EMM procedure showed significant time-space clustering of reported human brucellosis cases during the study period. Human brucellosis is not considered a contagious disease (2,19); therefore, clustering could result from common-source outbreaks or time-space clustering of factors that increase risk of infection. Human brucellosis is often associated with work-related (18,21,22) and foodborne (5,27) outbreaks, both of which were reported in California during 1973–1992 (25). The EMM procedure demonstrated clustering in total cases as well as Hispanic and non-Hispanic segments of the population but could not distinguish between risk-factor and common-source causes. Clustering in the Hispanic population was most evident in Los Angeles and other southern California counties during the study period. Non-Hispanic cases also tended to cluster in southern California, but not to the same degree as observed for Hispanics.

Spatial clustering of brucellosis incidence found by the Moran's I technique during 1973–1977 was significant even after the data were adjusted for age and race structure of county populations. This finding suggests that, during this 5-year period, clustering of cases did not result simply from spatial distribution of Hispanic populations. Until recently, brucellosis had been reported to be most strongly associated with occupation; farm workers, veterinarians, and meatpacking employees were at highest risk. Counties with the highest adjusted incidences during this period were those with a high degree of agricultural activity, suggesting the importance of traditional exposures.

Crude incidences of county-level human brucellosis were significantly clustered for 1983–1987 based on Moran's I. However, adjusting proportions for underlying race distributions removed the clustering, suggesting that clustering of human brucellosis cases during this time reflected the distribution of the Hispanic population. These findings confirm other reports that the epidemiology of human brucellosis is shifting from a disease of certain occupational groups to a foodborne

Table 1. Ederer-Myers-Mantel (EMM) procedure for time-space clustering of reported human brucellosis cases, California, 1973–1992

Population	No. of cases	Chi square[a]	p value[b]
Hispanic	305	3.800	0.03
Non-Hispanic	111	6.078	0.007
Total	416	8.100	0.002

[a]EMM test statistic follows an approximate chi-square distribution with 1 degree of freedom.
[b]p value based on a one-sided test (half of usual chi-square p value).

Table 2. Ederer-Myers-Mantel (EMM) procedure for time-space clustering of reported human brucellosis cases, California, 1973–1992

Years	Population	County	California area[a]	Chi square	p value[b]
1973–1977	Hispanic	Los Angeles	Southern California	74.94	<0.001
		Orange	Southern California	4.57	0.02
		Stanislaus	Central Valley	2.99	0.04
1978–1982	Hispanic	San Luis Obispo	Southern California	5.83	0.008
		Santa Barbara	Southern California	3.31	0.03
		Fresno	Central Valley	2.99	0.04
		Kern	Southern California	2.99	0.04
1983–1987	Non-Hispanic	Sacramento	Central Valley	2.99	0.04
		Santa Cruz	San Francisco Bay	2.99	0.04
1988–1992	Hispanic	Alameda	San Francisco Bay	15.42	<0.001
		Ventura	Southern California	11.95	<0.001
	Non-Hispanic	Los Angeles	Southern California	4.57	0.02
		San Bernardino	Southern California	2.99	0.04

[a]Southern California = Imperial, Kern, Los Angeles, Orange, Riverside, San Bernardino, San Diego, San Luis Obispo, Santa Barbara, and Ventura Counties; Central Valley = Colusa, Fresno, Glenn, Kings, Madera, Merced, Sacramento, San Joaquin, Solano, Stanislaus, Sutter, Tulare, and Yolo Counties; San Francisco Bay = Alameda, Contra Costa, Marin, San Francisco, San Mateo, Santa Clara, and Santa Cruz Counties.
[b]p-value based on one-sided chi-square test with 1 degree of freedom.

disease of the general population, with Hispanics at greatest risk (23,25).

Reported cases of human brucellosis were significantly clustered in all four 5-year periods based on total population I_{pop} analysis. Reported cases in Hispanics were also significantly clustered in all 5-year periods except 1983–1988. Results also show that most clustering was due to the number of cases within counties, rather than in adjacent counties. This finding was most apparent in the Hispanic-specific analysis for 1988–1992. More than 100% of the clustering effect was estimated for cases in the same counties. Cases in adjacent counties during this period were dispersed, driving the test statistic in the other direction (negative clustering effect). Clustering in specific counties was strong enough to overcome this dispersion effect. Brucellosis incidences in non-Hispanics of California were not clustered at any time during the study period.

The I_{pop} analysis suggests that occurrence of human brucellosis in non-Hispanics of California is a random event that does not appear to cluster in certain counties or regions. In contrast, Hispanic cases showed a strong tendency to be clustered in certain counties. This tendency was especially true for 1988–1992, when nearly 50% (48/97) of Hispanic cases were

reported in three nonadjacent California counties: Los Angeles (23 cases), San Diego (14 cases), and Alameda (11 cases).

Identification of spatial clustering of human disease for specific *Brucella* species can provide important epidemiologic information about animal reservoir and source of infection. Human infection due to *B. abortus* would be expected to cluster in counties with increased livestock activity early in the study period, when *B. abortus* was still endemic on some farms. Lack of significant clustering for 1973–1977 may have resulted from the small number of confirmed *B. abortus* infections and the resulting low statistical power for spatial tests. Spatial clustering of *B. melitensis* associated with Hispanic populations would be expected to be consistent throughout the 20-year study period. This observation held true except for 1983–1987, when no significant clustering was found for brucellosis cases in Hispanics or for *B. melitensis*-specific cases.

Data were analyzed by both the unadjusted and population-adjusted Moran's I techniques, because of known characteristics of human brucellosis. The I_{pop} analysis is more powerful than the unadjusted Moran's I (31), so the I_{pop} procedure is recommended when both numerator and denominator data are available. However, the null hypothesis of this statis-

Table 3. Moran's I procedure for spatial clustering of the four, 5-year cumulative incidences of reported human brucellosis, California, 1973–1992

Years[a]	No. of cases	Crude incidence[b]		Race adjusted[b]		Age-race adjusted[b]	
		z-score[c]	p value	z-score[c]	p value	z-score[c]	p value
1973–1977	106	2.31	0.02	2.75	0.006	2.84	0.004
1978–1982	97	1.07	0.29	0.05	0.96	-0.34	0.74
1983–1987	98	4.62	<0.001	0.31	0.75	0.27	0.79
1988–1992	115	-0.32	0.75	-0.74	0.46	-0.49	0.63
1973–1992 (mean)	416	1.30	0.19	-0.56	0.57	-0.26	0.79

[a]Cases summed over the specified 5-year period. Incidence over the 20-year period is the mean of incidences for the 5-year periods.
[b]Incidences are based on the population of the 5-year period's median year as the denominator. Incidences were adjusted by the direct method with the population distribution of Sacramento County in 1990 as the standard. Data for race were adjusted by using Hispanic and non-Hispanic population totals.
[c]Positive z-score indicates tendency towards clustering, negative value dispersion.
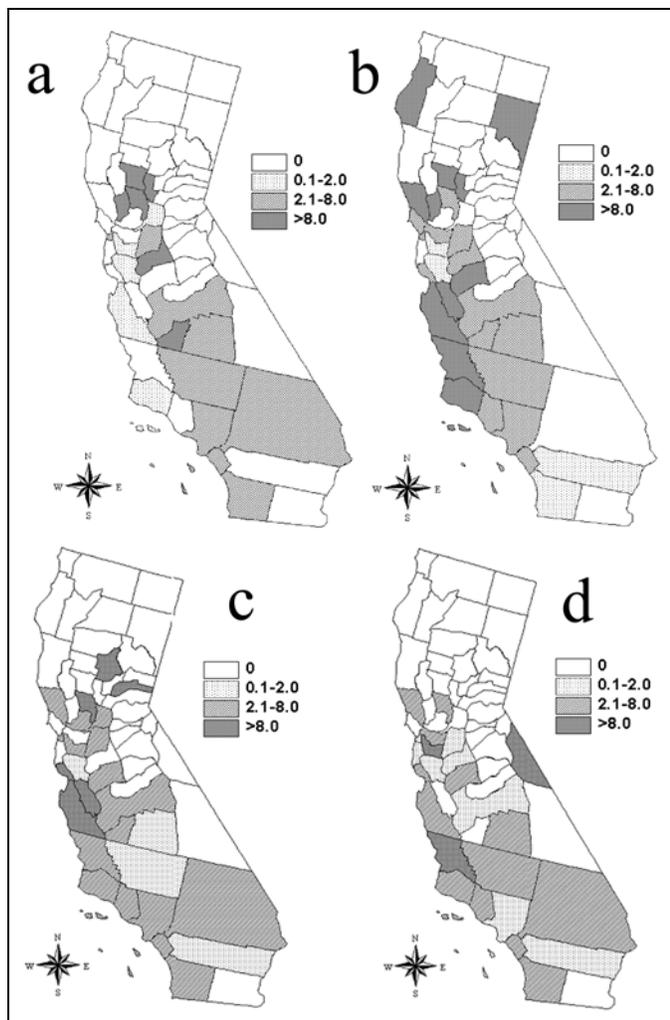
Figure 4. Distribution of human brucellosis: age/race-adjusted incidence per 10^6 population in California for the following 5-year periods: (a) 1973–1977, (b) 1978–1982, (c) 1983–1987, and (d) 1988–1992.

tical procedure is that cases are independent occurrences of disease in the underlying population at risk. Rejection of this null hypothesis leads to acceptance of the alternate hypothesis that clustering in disease occurrence is present. Human brucellosis is known to cluster in occupational and foodborne settings. Therefore, the statistical test may be biased because it is not able to subtract the effect of these outbreaks from the overall test statistic. This bias could be controlled if data were available for all outbreaks that occurred during the study period. The unadjusted Moran's I technique has lower power and is therefore more conservative than the I_{pop}. The true nature of human brucellosis clustering in California during 1973–1992 most likely falls somewhere between the results of these two statistical analyses.

Human brucellosis continues to be a major public health concern in California even though the United States has effectively reduced the level of *Brucella* infection in domestic animals (32). Our results suggest that the epidemiology of risk factors for human infection due to *Brucella* spp. in California has changed. More traditional sources of infection are less

important than the increased risk of *Brucella* spp. as foodborne pathogens. Traditional clustering of cases in concentrated agricultural regions was observed only for 1973–1977.

The Hispanic segment of the California population is at higher risk for disease due to *Brucella* infection than all other ethnic groups. Counties with high crude incidences of brucellosis correspond to those with large Hispanic populations. Increased risk has been attributed to certain dietary preferences, particularly for Mexican soft cheeses (23,25–27). Results suggest that time-space clustering of human brucellosis cases in California during the study period was predominantly due to clustered distribution of Hispanics in the state. However, I_{pop} and EMM results indicate residual temporal-spatial clustering of Hispanic cases in certain counties after the data were controlled for race through Hispanic-specific calculations. Counties with the highest adjusted incidences of human brucellosis during 1988–1992 were San Luis Obispo, Mono, and Alameda Counties. Only a single case was reported in Mono County during 1988–1992, but the small population size resulted in a relatively large incidence proportion.

Public health programs should focus on educating the Hispanic segment of the California population about the risks of consuming certain dairy products, such as soft cheeses, made from unpasteurized milk. Education must also extend to health-care providers who work in areas with large Hispanic populations likely to be exposed to illegally imported dairy products from areas where *Brucella* infection is still common in domestic animals. Efforts should be focused in southern California and San Francisco Bay area counties with the

Table 4. Population-adjusted Moran's I (I_{pop}) analysis for spatial clustering of reported human brucellosis cases, California, 1973–1992

Years	No. of cases	z-score	p value	Within %[a]	Among %[a]
Total population					
1973–1977	106	7.79	<0.001	75.4	24.6
1978–1982	97	9.04	<0.001	62.1	37.9
1983–1987	98	4.94	<0.001	56.5	43.5
1988–1992	115	4.22	<0.001	93.1	6.9
1973–1992	416	18.67	<0.001	58.6	41.4
Hispanic population					
1973–1977	60	6.84	<0.001	77.6	22.4
1978–1982	73	11.66	<0.001	61.2	38.8
1983–1987	75	1.19	0.23	78.5	21.5
1988–1992	97	3.87	<0.001	107.9†	-7.9[b]
1973–1992	305	15.18	<0.001	70.8	29.2
Non-Hispanic population					
1973–1977	46	1.52	0.13	80.7	19.3
1978–1982	24	0.15	0.88	95.0	5.0
1983–1987	23	1.47	0.14	91.0	9.0
1988–1992	18	0.49	0.62	82.9	17.1
1973–1992	111	1.41	0.16	92.3	7.7

[a]Percentage of estimated spatial clustering attributed to cases in the same counties and in adjacent counties.
[b]All identified clustering attributed to cases in the same counties. Negative value in % demonstrates dispersion of cases in adjacent counties.

Table 5. Population-adjusted Moran's I (I_{pop}) analysis for spatial clustering of reported human brucellosis cases due to *Brucella abortus* and *B. melitensis* in Hispanic populations, California, 1973–1992.

Years	No. of cases	z-score	p value	Within %[a]	Among %
B. abortus[b]					
1973–1977	12	-0.01	0.99	96.7	3.3
1978–1982	12	4.28	<0.001	74.8	25.2
1983–1987	9	-0.47	0.64	125.4[c]	-25.4[c]
1988–1992	23	0.15	0.88	108.8[c]	-8.8[c]
B. melitensis					
1973–1977	17	6.47	<0.001	62.4	37.6
1978–1982	48	8.77	<0.001	70.8	29.2
1983–1987	56	1.18	0.24	83.2	16.8
1988–1992	50	3.15	0.002	104.6[c]	-4.6[c]

[a]Percentage of estimated spatial clustering attributed to cases in same counties and in adjacent counties.
[b]Cases are reported as *B. abortus* or *B. melitensis* based on bacterial isolation or reported animal contact as cattle or goats, respectively, when bacterial isolation was not performed or species not determined.
[c]All identified clustering due to cases in the same counties. Negative value for in % demonstrates dispersion of cases in adjacent counties.

highest brucellosis incidences and absolute number of reported cases. More research focusing on the epidemiology of human brucellosis in California is necessary to aid in protection of its residents from disease.

Acknowledgments

Dr. Fosgate is a veterinarian and has received his PhD in epidemiology at the University of California, Davis. He has worked in private veterinary practice in the United States and has studied brucellosis in Nepal and Trinidad and Tobago. His research interests include diagnosis and control methods for *Brucella abortus* infection in species other than cattle.

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Address for correspondence: Geoffrey T. Fosgate, Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA 95616, USA; fax: 530-752-0414; e-mail: gtfosgate@ucdavis.edu

Persistent High Incidence of Tuberculosis in Immigrants in a Low-Incidence Country

Troels Lillebaek,* Åse B. Andersen,† Asger Dirksen,‡ Else Smith,* Lene T. Skovgaard,§ and Axel Kok-Jensen‡

Immigration from areas of high incidence is thought to have fueled the resurgence of tuberculosis (TB) in areas of low incidence. To reduce the risk of disease in low-incidence areas, the main countermeasure has been the screening of immigrants on arrival. This measure is based on the assumption of a prompt decline in the incidence of TB in immigrants during their first few years of residence in a country with low overall incidence. We have documented that this assumption is not true for 619 Somali immigrants reported in Denmark as having TB. The annual incidence of TB declined only gradually during the first 7 years of residence, from an initial 2,000 per 100,000 to 700 per 100,000. The decline was described by an exponential function with a half-time of 5.7 (95% confidence interval 4.0 to 9.7) years. This finding seriously challenges the adequacy of the customary practice of screening solely on arrival.

In most industrialized countries, the annual numbers of cases and deaths caused by tuberculosis (TB) have steadily declined over the past century up to the mid-1980s (1,2) (Figure 1). Since then, an increasing number of TB cases in immigrants has reversed this downward trend in countries that have had substantial levels of immigration from areas with a high prevalence of the disease (1,3,4) (Figure 2). Today, the proportion of immigrants among persons reported as having TB exceeds 50% in several European countries, including Denmark, Israel, the Netherlands, Norway, Sweden, and Switzerland (5). A similar proportion has been predicted for the United States in 2002 (3). In Denmark, the doubling in reported TB cases over the last 15 years has reflected, in large measure, TB in immigrants from Somalia (6), who also account for a sizeable proportion of TB cases in other European countries and North America (7–11).

The epidemiologic importance of migration for TB low-incidence countries has been recognized for several years; the main countermeasure has been the implementation of screening programs for immigrants at the time of arrival (4,12). In 1994, 20 of 23 European countries were reported to screen for TB on immigrants' arrival (4). This measure is based on the fundamental assumption of a prompt decline in the incidence of TB in immigrants from an area of high incidence during their first few years of residence in the country of low incidence. However, only a few studies have actually addressed this question (13–17).

To evaluate the implications for the practice of screening on arrival, we explored the changes with time of residence in the incidence of TB in immigrants from high-incidence areas. Our study focused on the 13,535 Somalis who arrived in Denmark during the 1990s, 901 of whom were subsequently reported as having TB.

Methods

The study was designed as a nationwide retrospective cohort analysis of surveillance data on all 901 Somalis reported as having TB in Denmark from 1991 to 1999. In Denmark, TB reporting has been mandatory since 1905. Since 1922, all cultures for mycobacteria have been performed at the International Reference Laboratory of Mycobacteriology at Statens Serum Institut in Copenhagen (18). During the study period, this laboratory provided bacteriologic data on all Somalis reported in Denmark as having TB. TB treatment is centralized in departments of respiratory, pediatric, and infectious medicine, which are also responsible for reporting data on all new and recurrent cases of TB by means of a standardized form. These individual reports are collected in a national surveillance register at the Department of Epidemiology at Statens Serum Institut. This department provided information
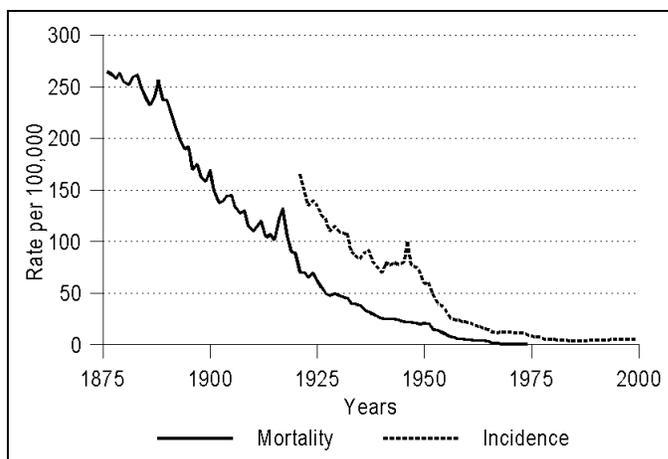
Figure 1. Cases of pulmonary tuberculosis in Denmark over a 125-year period, based on national surveillance information: mortality rates and incidence.

*Statens Serum Institut, Copenhagen, Denmark; †Rigshospitalet (National Hospital), Copenhagen, Denmark; ‡Gentofte University Hospital, Hellerup, Denmark; and §University of Copenhagen, Copenhagen, Denmark
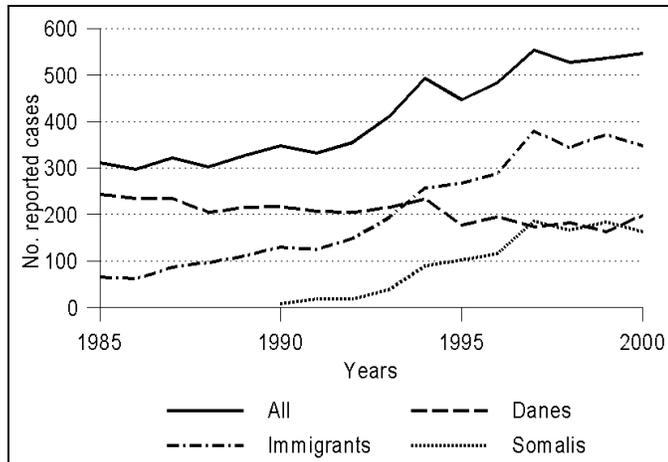
Figure 2. Trends in number of reported cases of tuberculosis in Denmark over the last 15 years, by nationality.

on the annual number of cases, nationality of the patients, and their date of entry into the country. The total number of Somalis in Denmark at the end of each year was taken from the Statistical Yearbooks from 1992 to 2000, published in Copenhagen by Statistics Denmark. The study was approved by the local medical ethics committees (No. 11-087/99) and the Danish Data Protection Agency (No. 2001-41-1018).

The following operational terms were used, adapted to Danish administrative terminology (4). A foreign-born person was any person born outside Denmark, while the term Somali was applied to any person born in Somalia. An immigrant was any foreign-born person legally admitted to Denmark who had already settled in the country or was expected to do so. A refugee was any person who had been granted refugee status by the Danish authorities. An asylum-seeker was any person wishing to be admitted to the country as a refugee but awaiting decision on his or her application for refugee status. An illegal

immigrant was defined as any person whose entry, stay, or work in the country was not permitted by the Danish authorities. Illegal immigrants are considered rare in Denmark, and no patient included in the study fell into this category. The word "screening" was used in connection with any interventions performed to discover *Mycobacterium tuberculosis* infection suitable for early preventive or curative therapy, in a person whose symptoms were not so severe as to cause him or her to seek medical help. The term "medical evaluation" was used in connection with interventions not specifically performed to discover *M. tuberculosis* infection, e.g., general health examinations.

Over the study period, the number of Somali immigrants in Denmark increased considerably, from 743 in 1991 to 14,856 in 2000 (Table 1). Because of this sustained rise, the annual increase in the number of Somali immigrants was classified as net arrival in Denmark, without taking into account the small numbers who were born, died, or left the country during the study period. For example, at the beginning of 1992 and 1993, respectively, 1,395 and 2,237 Somali immigrants were living in Denmark, corresponding to a net arrival in 1992 of 2,237 – 1,395 = 842 persons (Table 1). To compare changes in numbers from year to year, the observation period for all Somali immigrants included in the study in a given year was totaled as person-observation years (Table 1). The Somali immigrants entered Denmark at various times of the year; therefore, on average each immigrant contributed only a half person-observation year in the calendar year of arrival. For example, in 1992 a total of 1,395 Somali immigrants were already living in Denmark at the beginning of the year. They were observed during the whole calendar year and thus accounted for 1,395 person-observation years. A further 842 Somali immigrants arrived during 1992 and thus accounted for 842/2 = 421 person-observation years, if an even distribution of arrivals is

Table 1. Number of Somalis in Denmark and cases of Somali immigrants reported as having tuberculosis, 1991–2000

Year	a Somalis in Denmark[a] (no.)	b Net arrival[b] (no.) $(a_{n+1}-a_n)$	c Person-observation years yrs $(a + 1/2b)$	d Reported cases[c] (no.)	e Crude rate[d] (d as % of c) (95% CI)
1991 and earlier	743	652	1,069	NA	NA
1992	1,395	842	1,816	20	1.1 (0.7 to 1.7)
1993	2,237	1,552	3,013	41	1.4 (1.0 to 1.9)
1994	3,789	1,491	4,535	92	2.0 (1.7 to 2.5)
1995	5,280	1,811	6,186	100	1.6 (1.3 to 2.0)
1996	7,091	2,794	8,488	114	1.3 (1.1 to 1.6)
1997	9,885	2,228	10,999	182	1.7 (1.4 to 1.9)
1998	12,113	1,422	12,824	167	1.3 (1.1 to 1.5)
1999	13,535	1,321	14,196	185	1.3 (1.1 to 1.5)
2000	14,856	NA	NA	NA	NA

[a]Number of Somalis in Denmark by January 1.
[b]Estimated number of Somalis arriving in Denmark in 1 year.
[c]Somalis reported as having tuberculosis (TB).
[d]Crude incidence rate as % of the accumulated number of Somalis in Denmark; NA = not available; 95% CI = 95% confidence interval.

assumed throughout the year. Thus, the total number of Somali immigrants in 1992 was 1,395 + 421 = 1,816 person-observation years (Table 1). Calendar years of arrival and diagnosis were used because exact dates were not available.

In calculating incidences of TB in relation to duration of residence in Denmark, we gave special attention to Somalis reported as having TB during the period 1995–1999 (Table 2). Each incidence was calculated from the number of Somalis reported as having TB after a given number of years of residence in Denmark, divided by the total number of Somali immigrants who had resided in Denmark for the same number of years. In total, 748 Somalis were reported as having TB from 1995 to 1999 inclusive. Of these, 84 persons were excluded because of lack of information about their exact year of arrival in Denmark, in addition to 45 persons who were known to have arrived in 1991 or earlier (Table 2). Hence, we were able to calculate the risk for developing TB in relation to the average duration of residence in Denmark for 619 Somalis. For example, 158 Somalis were reported as having TB during their second calendar year of residence in Denmark, after an average of 1 year's residence in Denmark (Table 3). This number represents the sum of the 24 Somalis who arrived in 1994 and were reported in 1995, the 24 who arrived in 1995 and were reported in 1996, the 58 who arrived in 1996 and were reported in 1997, the 34 who arrived in 1997 and were reported in 1998, and the 18 who arrived 1998 and were reported in 1999 (Table 2). The TB incidence was then calculated by dividing by the person-observation years: the 158 Somalis who were diagnosed with TB during their second calendar year of residence in Denmark were found among 9,746 person-observation years for persons who on average had resided in Denmark for 1 year, giving an incidence of 158/9,746 = 1.6% (Table 3).

Table 2. Somali immigrants in Denmark reported as having TB, 1995–1999, by year of arrival and diagnosis

| Year | Total arrivals | Year of diagnosis (no./yr) | | | | | |
		1995	1996	1997	1998	1999	Total
1991 and earlier	1,395	8	5	10	8	14	45
1992	842	7	10	7	7	13	44
1993	1,552	30	13	10	18	10	81
1994	1,491	24	22	19	22	20	107
1995	1,811	17	24	29	29	21	120
1996	2,794	-	28	58	31	27	144
1997	2,228	-	-	21	34	19	74
1998	1,422	-	-	-	10	18	28
1999	1,321	-	-	-	-	21	21
Unknown yr of arrival (%)		14	12	28	8	22	84 (11.2)
Total TB[a] (%)		100	114	182	167	185	748 (100.0)

[a]Total number of Somali immigrants reported as having tuberculosis (TB).

Table 3. Risk for tuberculosis (TB) related to duration of residence in Denmark for Somali immigrants, 1995–1999

| Average residence[a] (yrs) | Person-observation years | Reported TB cases | | |
		No.	Incidence % (no. in % of yrs) (95% CI)[b]	Cumulated annual incidence (%)
1/2[b]	4,788[b]	97	2.0 (1.7 to 2.5)	1.0[b]
1	9,746	158	1.6 (1.4 to 1.9)	2.6
2	9,876	131	1.3 (1.1 to 1.6)	3.9
3	8,490	95	1.1 (0.9 to 1.4)	5.0
4	5,696	63	1.1 (0.9 to 1.4)	6.1
5	3,885	45	1.2 (0.9 to 1.6)	7.3
6	2,394	17	0.7 (0.4 to 1.2)	8.0
7	842	13	1.5 (0.9 to 2.7)	9.5

[a]Average duration of residence in Denmark
[b]Person-observation years and cumulated incidence only "counts half" in year after arrival (see Methods); 95% CI, 95% confidence interval.

For statistical analysis, 95% confidence intervals (CI) were derived from the normal approximation to the binomial distribution (Tables 1 and 3). The single p value given in the results was calculated by the chi-square test. If one assumes a Poisson distribution and an exponential decrease in incidence with time, the half-time of the decline in the observed incidences with time of residence was estimated (with 95% CI) by means of SAS statistical software (GENMOD procedure; SAS Institute Inc., Cary, NC).

Results

Basic Cohort Data

From 1991 to 1999 in Denmark, 4,147 persons were reported as having TB. Of reported patients, 57.5% (2,386/4,147) were foreign-born, of whom 37.8% (901/2,386) were Somali. For each year, 80%–91% of reported patients were culture positive for *M. tuberculosis*. Of total culture-positive patients, 74.7% had pulmonary TB with or without extrapulmonary disease, and 25.3% had extrapulmonary disease only. Foreign-born patients had a higher frequency of exclusively extrapulmonary TB than Danish patients (45.6% vs. 16.6%; p<0.001). Of Danish and foreign-born patients with culture-positive pulmonary TB, 55.3% and 26.2%, respectively, had sputum smears positive for acid-fast bacilli.

Trends in TB Incidence Related to Duration of Residence

The overall annual incidence rate for Somalis remained fairly steady at 1.1%–2.0% (Table 1), but when duration of residence in Denmark was taken into account, the incidence gradually decreased from 2.0% (CI 1.7 to 2.5) during the year of arrival to 0.7% (CI 0.4 to 1.2) during the sixth year of residence (Table 3; Figure 3). The only gradual decrease in incidence rate was described by a simple exponential model with a half-time of 5.7 (CI 4.0 to 9.7) years (Figure 3). Analysis of
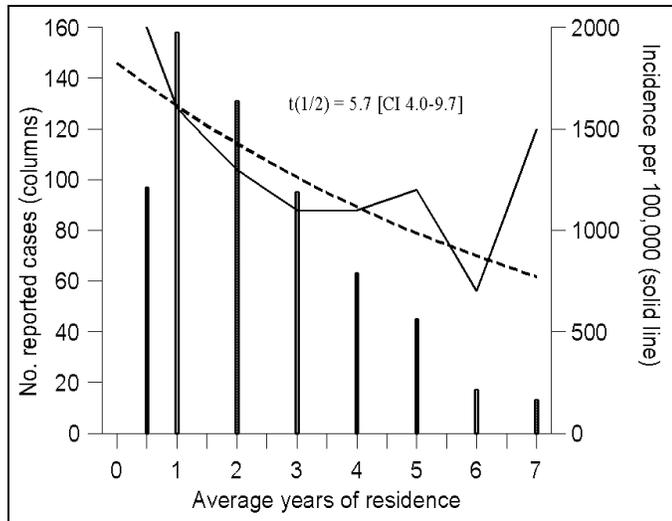
Figure 3. Trend in the incidence of tuberculosis in Somali immigrants in Denmark, by duration of residence. The dotted line indicates the estimated incidence curve and t(1/2) the corresponding half-time, with confidence interval.

residuals plotted against duration of residence, year of arrival, year of diagnosis, and person-observation years showed no obvious deviation from a simple exponential model during the first years of residence. During the seventh year of residence, the incidence increased to 1.5% (CI 0.9 to 2.7); however, as seen from the wide CI, this figure is subject to considerable uncertainty. Only 842 Somali immigrants, of whom 13 were reported as having TB, had been living in Denmark long enough to be eligible for observation during their seventh year of residence (Table 3). Overall, 9.5% of all Somalis who arrived in Denmark were diagnosed with TB during their first 7 years of residence (Table 3).

Discussion

TB after Arrival in a Low-Incidence Country

Our data show that the initial incidence of TB in Somalis entering Denmark was high, and more importantly, that this high initial incidence declined only gradually, at first in an exponential manner, in the 7 years after arrival. The incidence of TB in Somalis in Denmark is higher than in any other foreign-born population group in the country (19,20) and is comparable with or even higher than the estimated incidence in Somalia (21). During their first 2 years of residence in Denmark, 3.9% of all Somalis were diagnosed with TB, and after 7 years, 9.5% were reported as having TB (Table 3). The exact reasons for the extraordinarily high and only slowly declining incidence of TB in Somalis in Denmark remain unknown, but some theoretical possibilities are discussed in this section.

Epidemiologically, the most important potential impact of excess TB cases due to immigrants from an area of high incidence would be an increase in the rate of transmission in the recipient country of low incidence (22). However, the number of reported TB cases in the Danish-born population has not yet shown any detectable increase in parallel with the increase in

the cases in the foreign-born population (Figure 2). Moreover, a nationwide study of *M. tuberculosis* DNA patterns from 3,320 TB patients in Denmark recently indicated that TB due to transmission among Somalis in Denmark was limited, and transmission between Somalis and Danes was almost nonexistent (6). Recent *M. tuberculosis* transmission among the Somalis in Denmark cannot explain the high and only gradually declining incidence of the disease after arrival; furthermore, the Somalis in Denmark have not substantially increased their risk for TB infection, as they are diagnosed and treated promptly.

In combination with a high prevalence of dormant *M. tuberculosis* infection, impairment of the immune system (e.g., as a result of HIV infection) could reactivate latent disease (23,24). Indeed, *M. tuberculosis* bacteria in immigrants from high-incidence areas may constitute a pool from which active TB could develop. Without taking BCG vaccination status into account, tuberculin skin testing of 300 Somalis in Denmark has indicated that 80%–90% of all adults (16–49 years of age) and 25% of all children were infected with *M. tuberculosis* at the time of arrival (unpub. data). However, only 1–2 Somali TB patients are found to be HIV positive every year. In addition, four studies in Somalia reported a very low prevalence of HIV infection, even among prostitutes attending a clinic for sexually transmitted diseases in the capital Mogadishu (21,25–27). At present, HIV infection does not seem to play an important role in the development of TB in Somalis.

The mechanisms behind the pattern of incidence of TB in Somalis in Denmark merit further exploration. One of the principal hypotheses is that the immigrant population contains many cases of latent infection with *M. tuberculosis* that later produce overt disease (28). Factors that could promote this reactivation, which should be identified and examined, include vitamin deficiencies, genetic constitution, and immune defects. In the preantibiotic era, the risk of reactivation of TB after recovery was extraordinarily high: annual relapse rates were 4.4% during the first 5 years and 1.6% during the next 5 years (2). The situation in Somalia and the refugee camps from which the immigrants have come may resemble the preantibiotic era in the high number of relapses and reactivations now being observed. In addition, many cases of TB may not have been identified in the refugee camps, or if they were diagnosed, patients may not have received proper treatment because of lack of resources (16). The discovery of such cases during screening on arrival in Denmark could explain the high initial incidence and why this rate could exceed the estimated rate in Somalia, where cases may remain unrecorded (21).

Implications for Policy of On-Arrival–Only Screening

Only a few studies have described the trend in TB incidence in immigrants over the years after their arrival from an area of high incidence (13–17). Three of these studies were restricted to immigrants arriving from Asia (14–16), and two covered only a short period of observation (16,17). The general finding was of a prompt decline in incidence during the

first few years of residence in the receiving country, although two studies reported an increased TB risk many years after arrival, as we observed for the Somalis in Denmark (13,16). The observation of a prompt decline in incidence has had a major influence on the countermeasures taken to prevent and control the disease in low-incidence countries. Nearly all low-incidence countries have implemented programs in which immigrants are screened only at the time of arrival (4,12): in 1994 20 of 23 European countries followed this practice (4).

In Denmark, all refugees and asylum seekers are encouraged to have a general medical evaluation (not specific for TB) only at the time of arrival in the country. Those who do not arrive as refugees or asylum seekers do not undergo systematic medical evaluations but are entitled to contact the free public health-care system on their own initiative. After the initial medical evaluation, the immigrants, refugees, and asylum seekers in Denmark, as in most other low-incidence countries, are covered by the national TB program, which is based on passive case-finding and treatment of active cases, combined with contact tracing (29). This program involves chest x-ray examination if pulmonary symptoms persist for >6 weeks, examination for *M. tuberculosis* if chest x-ray is suggestive of TB, examination by chest x-ray every 6 months for 3 years in tuberculin-positive subjects who have had recent exposure to a smear-positive TB patient, examination for *M. tuberculosis* from extrapulmonary sites if symptoms indicate TB, free four-drug short-course treatment regimens for TB patients, and preventive chemotherapy only for children <7 years of age whose tuberculin skin test is positive (29).

The gradually declining incidence in the years after arrival observed for Somalis in Denmark, persons of different nationalities in the United States (13), and Asians in Canada (16) seriously challenges the adequacy of the policy of screening only on arrival. National TB programs in low-incidence countries should be expanded to include surveillance of trends in the incidence of TB in specific immigrant populations during subsequent years as well. If a gradual decline similar to that in the Somalis in Denmark and Asians in Canada is observed, the present policy of screening only on arrival needs to be revised and refocused. Such revision would probably include as an important feature the institution of voluntary regular health examinations, at reasonable intervals after arrival, for specifically identified high-risk immigrant groups, as the risk may persist for many years. Intervention needs to be an ongoing process that includes both latent *M. tuberculosis* infection as well as active TB.

Another way of preventing TB in high-risk groups such as the Somalis in Denmark could be preventive chemotherapy, i.e., treatment of persons with subclinical *M. tuberculosis* infection. Several controlled studies have documented the effectiveness of such a strategy in preventing progression to TB or reactivation of disease on an individual basis (30), but the effectiveness of preventive chemotherapy administered to population groups needs further evaluation (22,31). The com-

pliance of participants is crucial for obtaining satisfactory results (30). For instance, a large meta-analysis showed that only 60.5% of 1,084,760 persons completed preventive therapy (32). If preventive therapy is used indiscriminately, a large number of infected persons would have to be treated to prevent the occurrence of a single case of TB (30), and all those treated would be at risk of side effects from the medication (32). However, preventive therapy may decrease illness for the 9.5% of Somalis who have TB during the first 7 years of residence in Denmark, if the medication is efficiently distributed to the Somalis with latent infection.

Focused Intervention: Key to Future Control?

As TB declines in low-incidence countries, *M. tuberculosis* transmission is markedly reduced, and most cases arise in persons who have previously been infected (3). Most cases of TB infection have been acquired in the same country, as has been observed for most older Danish-born TB patients, or have been acquired in another country where TB is still actively transmitted and subsequently been imported, as observed in the Somalis in Denmark (6). Thus, in low-incidence countries TB has increasingly come to be a disease of specific subgroups of the population (22). This trend provides an opportunity for focused intervention, the success of which will depend on correctly identifying the population groups at risk. Because of the considerable geographic variations in TB in immigrants from different countries and different trends in incidence after arrival in various host countries, approaches to controlling and preventing TB should be tailored to the specific foreign-born populations at risk. Control and elimination strategies should be focused on diminishing the incidence and prevalence of latent infection to reduce the pool of TB infection from which future cases of TB will emanate. This goal can be accomplished by two approaches: first, to reduce the incidence of new TB infection and thereby limit the growth of the pool and second, to reduce its prevalence (33). To arrest the chain of transmission, the risk of new generations becoming infected must be minimized by the early identification and curative treatment of newly emerging infective sources. Furthermore, newly infected persons must be prevented from progressing to overt disease; this approach reduces the number of cases caused by recent transmission (3). Our study also underlines the importance of transition from latent infection to active disease. If we seek to control the rates of TB in immigrants arriving from areas of high incidence, the success of our control measures will increasingly depend on reducing the impact of TB in immigrants by arresting the transition from latent to active disease. However, the global perspective of TB should also be kept in mind: the impact of disease falls principally on developing nations, where 95% of all cases and 98% of deaths due to TB occur (34). Intervention in such high-incidence areas, in addition to intervention in the low-incidence countries, is still crucial for the elimination of TB.

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Address for correspondence: Troels Lillebaek, Statens Serum Institut, International Reference Laboratory of Mycobacteriology, Artillerivej 5, DK-2300 Copenhagen S, Denmark; fax: 45 32 68 38 71; e-mail: tll@ssi.dk

Automatic Electronic Laboratory-Based Reporting of Notifiable Infectious Diseases at a Large Health System

**Anil A. Panackal,* Nkuchia M. M'ikanatha,† Fu-Chiang Tsui,‡ Joan McMahon,§
Michael M. Wagner,‡ Bruce W. Dixon,§ Juan Zubieta,* Maureen Phelan,* Sara Mirza,*
Juliette Morgan,* Daniel Jernigan,* A. William Pasculle,‡ James T. Rankin, Jr.,†
Rana A. Hajjeh,* and Lee H. Harrison‡**

Electronic laboratory-based reporting, developed by the UPMC Health System, Pittsburgh, Pennsylvania, was evaluated to determine if it could be integrated into the conventional paper-based reporting system. We reviewed reports of 10 infectious diseases from 8 UPMC hospitals that reported to the Allegheny County Health Department in southwestern Pennsylvania during January 1–November 26, 2000. Electronic reports were received a median of 4 days earlier than conventional reports. The completeness of reporting was 74% (95% confidence interval [CI] 66% to 81%) for the electronic laboratory-based reporting and 65% (95% CI 57% to 73%) for the conventional paper-based reporting system (p>0.05). Most reports (88%) missed by electronic laboratory-based reporting were caused by using free text. Automatic reporting was more rapid and as complete as conventional reporting. Using standardized coding and minimizing free text usage will increase the completeness of electronic laboratory-based reporting.

Public health surveillance of infectious diseases is crucial for detecting and responding to illnesses that may represent potential outbreaks or bioterrorism events (1,2). The Centers for Disease Control and Prevention (CDC) is collaborating with state health departments to improve current disease surveillance by using a standards-based information architecture through the National Electronic Disease Surveillance System (NEDSS), which includes electronic laboratory-based reporting of certain diseases to local, state, and federal public health authorities (3–5). Automatic reporting at private clinical laboratories in Hawaii has been shown to be more rapid and complete than conventional reporting (6).

Allegheny County (population 1,348,000) is located in southwestern Pennsylvania and includes the city of Pittsburgh. Incidences of notifiable diseases in the county are required by law to be reported directly to the Allegheny County Health Department (ACHD). Each notifiable event is recorded on a case report form that is mailed or faxed to the ACHD by laboratory personnel, physicians, nurses, or infection-control staff; this procedure constitutes the conventional paper-based reporting system (Figure 1). A notifiable event is considered reported when received and confirmed by the health department.

The UPMC Health System is a large university-based health-care network consisting of approximately 20 hospitals and hospital affiliates (http://www.upmc.edu) in western Pennsylvania and is affiliated with the University of Pittsburgh

School of the Health Sciences. UPMC established real-time, electronic laboratory-based reporting. This system is based on an existing hospital communications infrastructure designed to improve the speed and completeness of reporting (Wagner MM et al., unpub. data). ACHD personnel estimate that 40% of all notifiable infectious diseases reported to the ACHD come from UPMC. We evaluated the accuracy, completeness of coverage, and timeliness of electronic laboratory-based reporting before its integration into the conventional paper-based reporting system (7).

Background

Eight UPMC hospital microbiology laboratories in Allegheny County are capable of electronic laboratory-based reporting by using Health Level 7 (HL7), an electronic messaging standard for data exchange and communication between health-care information systems (http://www.hl7.org). Laboratory personnel and health-care providers who obtained results from these laboratories were required to report through the paper-based system and were unaware of the establishment of new electronic reporting (Figure 1). Once a laboratory technician obtained a test result, he or she entered the information into the hospital laboratory computer, which generated an HL7 message. Although laboratory workers could enter test results by using preprogrammed codes or free text (non-coded, non-standardized text entered by laboratory personnel), the electronic laboratory-based reporting system monitored only coded organism names in each HL7 culture message.

The processing occurred in real time, i.e., messages were checked as they were received. Instead of a batch mode in which data are extracted from sets of reports at predetermined

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †Pennsylvania Department of Health, Harrisburg, Pennsylvania, USA; ‡University of Pittsburgh, Pittsburgh, Pennsylvania, USA; and §Allegheny County Health Department, Pittsburgh, Pennsylvania, USA
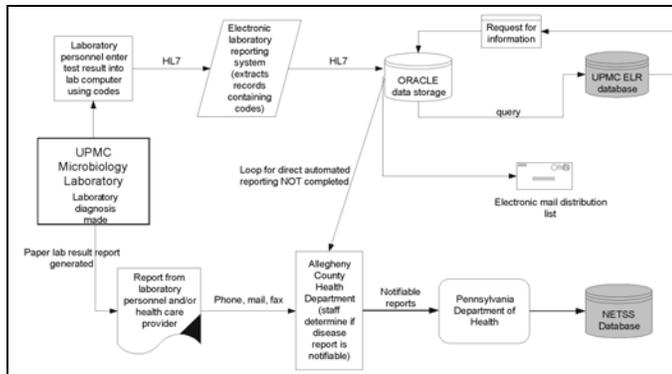
Figure 1. Schematic of information flow for the electronic reporting system of the UPMC Health System and for the paper-based reporting system to Allegheny County Health Department. NETSS refers to National Electronic Telecommunications System of Surveillance and ELR is electronic laboratory-based reporting.

times, extraction of information occurred whenever data were received by the electronic system. The electronic system extracted the specific laboratory specimen, procedure, and result from the HL7 records. The data were then interpreted; a laboratory result was positive or negative based on the code in the result section, which was compared with a data dictionary developed by the UPMC Health System. Some duplicate records were recognized by the electronic system. The extracted three- or four-letter coded organism name, defined by the UPMC laboratory system data dictionary, was then converted to its full name through a translation table maintained in the Oracle (Oracle Corporation, Redwood Shores, CA) data storage; computer personnel could use the Oracle data storage to add or remove an organism they wanted to be monitored. To obtain complete patient demographic information, if not provided in the laboratory HL7 message, the electronic laboratory-based reporting system queried the Medical Archival Retrieval System at UPMC based on the patient's medical record number in the message (8). Simultaneously, an electronic mail message containing the laboratory test result was sent by the electronic reporting system to selected UPMC personnel. Of note, the loop for direct automatic reporting between UPMC and ACHD was not completed at the time of this evaluation and, thus, no notifiable events were reported by the electronic system to the ACHD.

Methods

We conducted a comparison evaluation of the UPMC electronic laboratory-based reporting and the ACHD conventional paper-based reporting systems. From the eight UPMC hospital or affiliated microbiology laboratories with HL7 links in Allegheny County, we compared all disease reports in the UPMC electronic and the ACHD paper-based systems (derived from the National Electronic Telecommunications System of Surveillance) databases with dates of positive culture from January 1 to November 26, 2000, for 10 infectious organisms: *Campylobacter*, *Cryptosporidium*, *Escherichia coli* O157:H7, *Giardia*, *Listeria*, *Legionella, Neisseria meningitidis*, *Salmonella*, *Shigella*, and *Yersinia*. The diseases

caused by these organisms are notifiable to ACHD, requiring specific laboratory findings to meet the CDC case definition for notifiable diseases (9,10). Reporting of *Legionella* was evaluated for the period June 21–November 26, 2000, because the UPMC electronic laboratory-based reporting did not capture reports of diseases caused by this organism before June 21. Duplicate records and cultures performed in the context of research studies not notifiable to ACHD but included in the UPMC electronic database were excluded. Case reports in each database were matched manually by the investigator. A match was defined as a report in the UPMC electronic database that had the same patient name, date of birth, and type of notifiable infectious disease as a report in the ACHD paper-based database. After matching, the case reports that were found in both databases, as well as cases found in only one of the two databases, were entered into a separate Excel (Microsoft Corp., Redmond, WA) spreadsheet.

Completeness of reporting was defined as the total number of unique, notifiable events identified independently through each surveillance system (UPMC electronic laboratory-based and ACHD conventional paper-based systems) divided by the estimated total number of reports available for reporting at the laboratory level (N) (Figure 2). To estimate the total number of reports available, we used the Chandra Sekar-Deming capture-recapture method (12). Since both the UPMC electronic and ACHD systems may not have captured all notifiable events,

R/N = Completeness of Coverage of Conventional Surveillance System
S/N = Completeness of Coverage of Electronic Surveillance System
$N = [(R + 1) (S + 1)/ (C + 1)] – 1$
$R = C + n1$
$S = C + n2$
$X = N – C – n1 – n2$
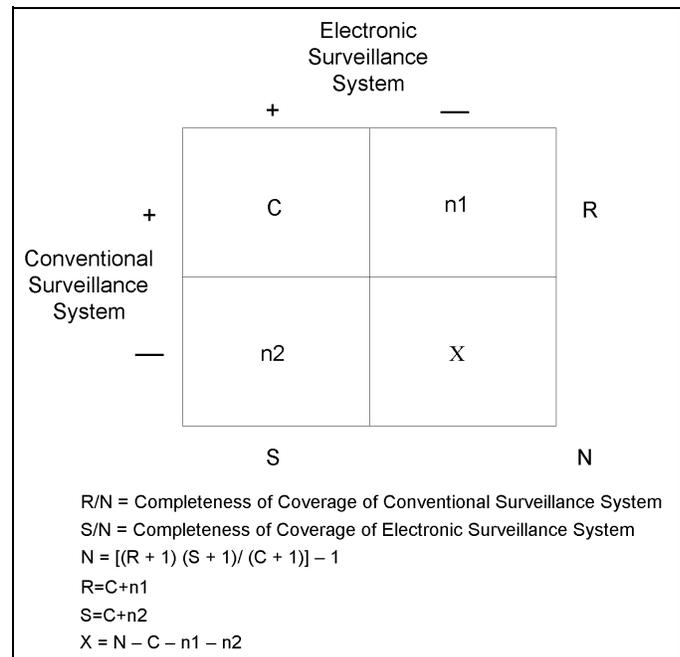
Figure 2. Capture-recapture methodology (11). C=number of reports received through both electronic laboratory-based reporting and conventional paper-based reporting. n1=number of reports received through conventional paper-based reporting system only. n2=number of reports received through electronic laboratory-based reporting only. X= estimated number of reports missed by both electronic laboratory-based reporting and conventional paper-based reporting system. R=number of reports received through conventional paper-based reporting system. S=number of reports received through electronic laboratory-based reporting. N=estimated total number of reports available by the Chandra Sekar-Deming capture-recapture calculation.

capture-recapture provided an approximation of the true total number of notifiable cases based on samples from these two independent, parallel surveillance systems (12,13). The overall completeness of reporting, the completeness of reporting by disease and by hospital, and the 95% confidence interval (CI) for completeness of coverage calculations were determined by using a resampling analysis based on the capture-recapture method (11). To date, no methods for the calculation of the 95% CI for completeness of coverage have been published. When SAS version 8.1 (SAS Institute, Inc., Cary, NC) was used, the resampling was based on the assumption that the distribution of the data observed for the three cells (e.g., C, n1, n2) of the contingency table followed a uniform distribution; the 5% and 95% values of the distribution from this analysis yielded the 95% CI for completeness. Completeness could not be calculated for diseases and hospital laboratories with zero values in the 2 by 2 contingency table cells (Figure 2).

An electronic false-positive result was defined as a case that was incorrectly detected by the electronic system; a missed report (or false negative) was defined as a notifiable case that was not detected through the electronic system. The completeness of reporting of both systems was estimated after excluding false positives and duplicate reports.

The chronologic sequence of events for the reporting of an infectious disease or condition consists of exposure to an infectious agent, followed by symptom onset after an incubation period, and then the seeking of medical attention (Figure 3). Although a presumptive diagnosis could be made by interpretation of the clinical syndrome at this point, the ability of electronic laboratory-based reporting system to detect a notifiable disease or condition begins at the time the laboratory result has been entered into the data system.

To determine the timeliness of the two surveillance systems, three time points were defined. T_1 was the date/time when the laboratory result was obtained and entered into the UPMC laboratory computer. T_2 was the date/time when the laboratory result was reported to ACHD by the conventional paper-based system. T_3 was the date/time the automatic electronic laboratory-based system notification was generated at UPMC. The timeliness of electronic and paper-based systems was defined as $t_3 - t_1$ and $t_2 - t_1$, respectively. The difference between t_3 and t_2 represented how much sooner or later the electronic system identified notifiable diseases than the paper-based system. Median differences were expressed with an interquartile range. The timeliness calculations were performed with SAS version 8.1.

Before matching individual records in both systems and removing duplicate records, we calculated the completion rates for the data fields common to both the UPMC electronic and the ACHD paper-based databases by using Epi-Info 2000 (Centers for Disease Control and Prevention, Atlanta, GA).

We identified the specific reasons for the electronic system's false positives and missed reports by using a traceback error analysis. Reports that were found in the UPMC electronic database but not in the ACHD paper-based database were identified, and case-patient information was reviewed from the laboratory computer reports and their HL7 messages (electronic false positives). Reports that were found in the ACHD paper-based database and not in the UPMC electronic database were identified after reviewing case-patient information, laboratory case reports, and archived computer files (electronic laboratory-based reporting missed reports). To further assess database accuracy, we also reviewed the paper reports and logs at the Allegheny County Health Department and compared these with data in the ACHD conventional paper-based reporting system database.

Results

A total of 141 unique reports were identified; 116 (82%) were reported through the UPMC electronic laboratory-based system, and 94 (67%) were reported by ACHD conventional paper-based reporting system. Forty-seven (33%) of the notifications were received through the UPMC electronic system only, 25 (18%) through the ACHD paper-based system only, and 69 (49%) through both (Figure 4). The estimated total number of reports calculated by the capture-recapture method was 144.

After excluding electronic laboratory-based reporting false positives, the overall completeness of reporting was 74% (95% CI 66% to 81%) for the UPMC electronic system and 65% (95% CI 57 to 73%) for the ACHD paper-based system (p>0.05), showing no significant difference in completeness of reporting between the electronic and paper-based systems (Table 1). Table 1 also lists the completeness of coverage and 95% CI by disease and by hospital. Most of the cases missed by electronic reporting were from one hospital (UPMC Hospital C).
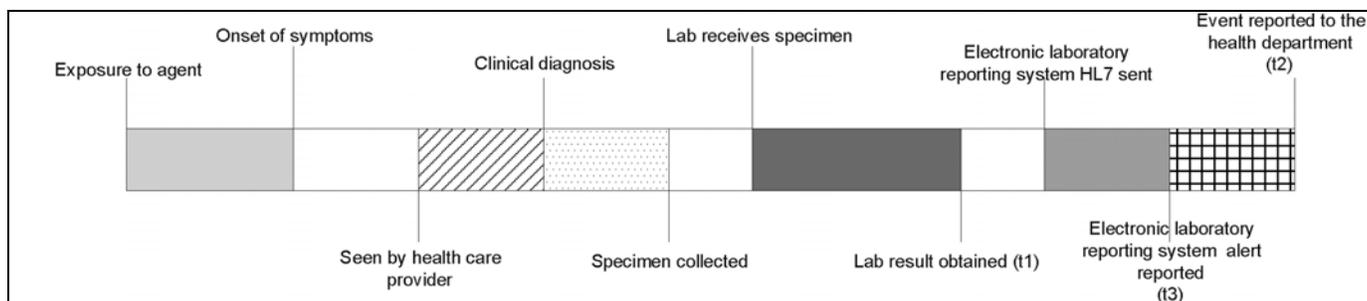
Figure 3. Timeline for reporting notifiable infectious diseases by the UPMC Health System, Pittsburgh, Pennsylvania, USA.
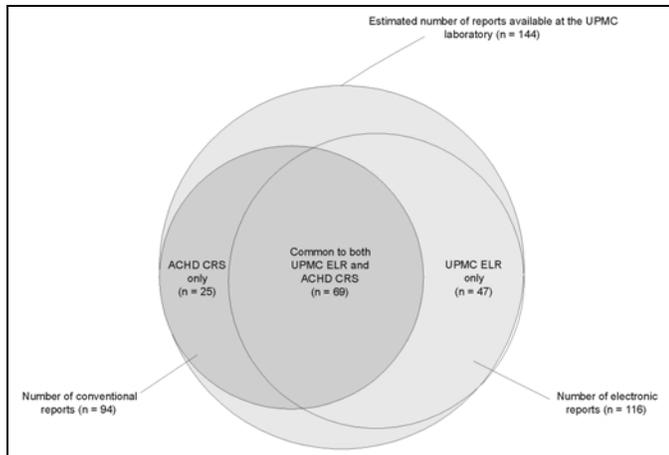
Figure 4. Venn diagram depicting the number of notifiable disease reports received independently by the electronic laboratory-based reporting of UPMC electronic system, Allegheny County Health Department paper-based reporting, or both. The estimated true total number of reports available, calculated by the Chandra Sekar-Deming capture-recapture method, is shown in the large, encompassing circle. ELR is electronic laboratory-based reporting, and CRS is conventional paper-based reporting system.

Timeliness was calculated by using the 69 records common to both databases. The timeliness of paper-based reporting was a median of 5 days (interquartile range 4 days). The timeliness of the electronic reporting was a median of 1 day (interquartile range 0 days). Electronic alerts were reported a median of 4 days (interquartile range 4 days) sooner than through paper-based reporting. We discovered a trend in the UPMC electronic reporting: the time difference between the date/time the laboratory result was obtained and entered, and the date/time the HL7 message was sent was almost exactly 24 hours to the second. After extensive discussions with the

UPMC laboratory administration and informatics personnel, the reasons for this finding are unknown.

Eleven data fields were common to both the UPMC electronic and the ACHD paper-based databases (Table 2). Of these, six fields were 100% complete in both. Of the remaining five, two were more complete in the UPMC electronic system (date of birth and age), whereas three were more complete in the ACHD paper-based system (address, zip code, report status [i.e. final results]); these differences were significant (p<0.001).

Electronic laboratory-based reporting generated 10 reports that were found to be false upon investigation (Table 3). Since the electronic reporting system can only capture diseases that are entered with the preprogrammed UPMC disease codes, test results entered with free text could not be extracted correctly into the UPMC electronic database. Most of the identified errors were, in fact, caused by the use of free text in combination with the UPMC code for the organism (i.e., the combining of the free text "No" with an organism code when laboratory technicians entered results). For example, a result entered by laboratory technicians as "no" "SALM" (the UPMC code for *Salmonella*) was recognized and incorrectly detected by electronic system as a positive case of *Salmonella*. Other errors involved the inability to retract preliminary reports of the isolation of notifiable organisms that were not subsequently confirmed and data extraction from the incorrect part of the result field. In the latter instance, a case of legionellosis was reported as listeriosis because the data field included the phrase "Specimen Delivery: UNIT LIST," and LIST is the disease code at UPMC for *Listeria*.

Data-entry errors, such as the incorrect use of free text, led to missed reports in the electronic system. Typically, these

Table 1. Completeness of coverage for UPMC electronic and conventional reporting systems by the notifiable infectious disease and hospital laboratory[a]

	Total no. of available reports[b]	Conventional reporting (ACHD)		Electronic reporting (UPMC)	
		No. of reports received	Completeness of coverage (95% CI)	No. of reports received	Completeness of coverage (95% CI)
Notifiable infectious disease					
Campylobacter	37	25	0.68 (0.49 to 0.85)	18	0.49 (0.32 to 0.65)
Salmonella	35	32	0.91 (0.83 to 0.97)	34	0.95 (0.91 to 0.97)
Escherichia coli O157:H7	17	10	0.59 (0.33 to 0.86)	7	0.41 (0.19 to 0.67)
Giardia	22	13	0.59 (0.39 to 0.77)	17	0.77 (0.58 to 0.90)
Neisseria meningitidis	9	5	0.58 (0.30 to 0.88)	7	0.72 (0.46 to 0.88)
UPMC Hospital laboratory					
A	26	16	0.62 (0.46 to 0.80)	24	0.92 (0.81 to 0.96)
B	52	29	0.55 (0.42 to 0.65)	47	0.91 (0.79 to 0.96)
C	35	24	0.69 (0.43 to 0.90)	9	0.26 (0.12 to 0.40)
D	13	11	0.85 (0.64 to 0.92)	12	0.87 (0.71 to 0.92)
E	10	9	0.90 (0.70 to 0.90)	9	0.90 (0.70 to 0.90)

[a]UPMC Health System; ACHD, Allegheny County Health Department; CI, confidence interval.
[b]Estimated total number of reports available by using capture-recapture (N in Figure 2).

Table 2. Data field completion rates on common data fields for cases in UPMC Health System electronic and conventional reporting system databases[a]

Data field	No. (%) of conventional reported cases with field completed (n=534)	No. (%) of electronic reported cases with field completed (n=582)
Patient information		
Patient ID	534 (100)	582 (100)
Name	534 (100)	582 (100)
Sex	534 (100)	582 (100)
Date of birth	462 (86.5)	582 (100)
Age	518 (97.0)	582 (100)
Address	533 (99.8)	306 (52.6)
Zip code	533 (99.8)	306 (52.6)
Specimen information		
Organism name	534 (100)	582 (100)
Time result obtained	534 (100)	582 (100)
Time result reported	534 (100)	582 (100)
Other information		
Status of report	534 (100)	220 (37.8)

[a]All rates before matching and duplicate record removal.

errors occurred when laboratory technicians entered the name of the organism as free text rather than with the preprogrammed UPMC disease codes. These errors accounted for 22 (88%) of 25 electronic missed reports, whereas the remaining three missed reports were found in the hospital computer systems but were not detected by the UPMC electronic system for reasons that remained unclear after investigation. Of the 47 cases in the UPMC electronic system not reported by the paper-based system to ACHD, 37 should have been reported to ACHD ("ACHD false negative").

Discussion

This is the first report of an evaluation of an existing health-system–based electronic notifiable disease reporting system. The electronic laboratory-based reporting was as complete as conventional paper-based reporting. The estimated completeness (74%) is similar to the recent report of 80% completeness of the electronic laboratory-based reporting from commercial clinical laboratories to the Hawaii Department of Health (6). The incompleteness and inaccuracy of UPMC electronic reporting were caused mainly by the use of free text, rather than standardized organism codes, by laboratory personnel at one hospital. Similarly, most of the electronic false positives were caused by the use of free text.

The magnitude of the difference in completeness between electronic laboratory-based reporting and conventional paper-based reporting may have been greater if it had been possible to review reports coming exclusively from laboratories to the ACHD; paper-based systems receive reports from sources other than laboratories. Data specifying if a case record originated from a laboratory or health-care provider were not available in the paper-based database. Hence, a bias favoring completeness of reporting by the paper-based system existed in our analysis. However, most reports received by health departments originate from clinical laboratories (14). The capture-recapture method used to calculate completeness required that the two surveillance systems (UPMC electronic laboratory-based reporting and ACHD conventional paper-based reporting system) operate independently. However, some interaction between the systems existed; the laboratory director used the generated electronic e-mail message, containing the laboratory test results, to check for potential false positives before a report was falsely sent conventionally to ACHD. This interaction was thought to be minimal (Figure 1). Other capture-recapture assumptions, such as the surveillance being performed on a stable population and only true matches and events being identified by the systems, were fulfilled (12,13).

Maximizing electronic laboratory-based reporting sensitivity is important for detecting diseases, while maximizing specificity enhances the likelihood that cases are reported correctly. Theoretically, electronic reporting has the potential to be both sensitive and specific, with few false negatives and false positives. The specificity of electronic reporting could be particularly high for diseases diagnosed by laboratory tests with a low rate of false positives (e.g., culture for enteric organisms); the diseases caused by the organisms used in this study met this qualification. Notifiable diseases based on other types of tests (e.g., serology for syphilis) would require clinical criteria to enhance specificity (information not available by electronic reporting). In this evaluation, we found that the inability to retract preliminary positive laboratory reports that were subsequently confirmed to be negative reduced the specificity of electronic reporting. However, in some instances, the benefit of early detection might supersede an occasional false-positive report. For example, early detection is paramount for some organisms, such as *Bacillus anthracis*—the release of which could represent a potential bioterrorist event. Nonetheless, a substantial amount of public health effort might be expended unnecessarily if such a laboratory finding were found to be a false positive. One must balance the tradeoffs between sensitivity, specificity, and timeliness when deciding to allow these preliminary laboratory results to be reported.

The UPMC electronic reporting has the potential to serve as a prototype for use nationally because it uses hospital-based laboratory information systems already in place to capture cases of disease that may be representative of the population at large. However, several findings from our analysis have implications for large health systems attempting to establish electronic laboratory-based reporting. The use of standardized disease codes should be encouraged because it maximizes both the sensitivity and specificity of electronic laboratory-based reporting. At UPMC, the incorrect use of free text at a single hospital substantially reduced the overall completeness of electronic laboratory-based reporting reporting. However,

Table 3. Electronic false positives and missed reports in UPMC Health System reporting system

Errors	No. (%) of electronic or paper-based only reports	Nature of problem
Electronic false positives		
Incorrect use of free text with organism codes	6 (60)	Culture report reads "No [free text]" followed by organism ID code
Inability to retrieve sent false reports	3 (30)	Unable to retrieve preliminary reports
Failure of logic detection	1 (10)	Data extracted from wrong portion of result field by logic detection
Total	10	
Electronic false negatives (missed reports)		
Incorrect use of free text	22 (88)	Organism name typed out as free text in result field
Unknown (failure of transmission?)	3 (12)	Found to be in UPMC hospital computer terminal system by using organism ID code properly but not found in UPMC electronic database
Total	25	

eliminating the use of free text may not be desirable from a laboratory personnel standpoint. As such, training laboratory personnel in the correct use of free text is important (15). Moreover, UPMC computer personnel could relegate the free text option only to a note field that provides useful information to health-care providers without generating a report; in this regard, a properly constructed result code entered through a correctly designed data entry method would be useful. The use of standardized codes has broad implications for electronic laboratory-based reporting in general. To effectively enhance the unification of NEDSS, CDC recommends the implementation of standardized coding schemes for disease names (Systematized Nomenclature of Medicine [SNOMED]; http://www.snomed.org) and laboratory test names (Logical Observation Identifier, Names, and Codes [LOINC]) (http://www.regenstrief.org//loinc/). Unfortunately, UPMC and many other health centers are not using standardized coding schemes. The lack of standardized coding requires the creation of a translation table, a process that requires refinements to maximize accuracy and completeness.

A mechanism for retracting of preliminary reports not subsequently confirmed is essential to reduce false-positive reports. Retraction is the ability to both remove an incorrect or preliminary report from the database as well as to notify the recipients of the information of the change. If the sending system does not explicitly label the message as a correction or a retraction, then the electronic laboratory-based reporting system must have logic to detect it. The detection logic simply compares the previously reported preliminary reports in a cached table with a new one. If the logic finds a match but the new report does not have any notifiable organisms, the logic will send a retraction alert to officials at the local health department or hospital laboratory administrators and remove the false-positive report from the cached table. At the time of this evaluation, such retraction capability did not exist at UPMC because the UPMC laboratory sending system did not explicitly label the message as a correction or a retraction. In

the future, one option may be to label preliminary reports as "preliminary" or "suspect." Currently, two authors have been working on the retraction capability and expect to have such functionality available soon. However, the risk versus benefit of reporting preliminary laboratory results should be weighed in making the decision to retract such reports. The best approach might be to report preliminary results for diseases that require immediate notification, while reporting confirmed results for others.

Decisions to remove certain duplicate records that were not detected by electronic laboratory-based reporting should be made before integration of automatic reporting to ACHD. Caution should be used in the removal of some type of duplicates, as this decision may need to be disease specific. For example, repeated positive sputum cultures for tuberculosis from a patient who has received the recommended course of therapy may represent persistent, active infection and drug resistance, both of which are substantial public health concerns.

UPMC electronic laboratory-based reporting had lower completion rates of data fields with important contact information (specifically, address and zip code fields) compared with ACHD conventional paper-based reporting system, which may hamper efforts by public health personnel to contact patients quickly. The implementation of automatic demographic data extraction by electronic laboratory-based reporting from other resources such as epidemiologic or administrative databases (e.g., billing records), could substantially improve the data field completion rate for electronic laboratory-based reporting.

UPMC should continue to refine its electronic laboratory-based reporting before implementing direct automatic reporting to ACHD. Electronic laboratory-based reporting should not replace conventional reporting as observations made by astute clinicians are valuable in the timely reporting of certain notifiable syndromic illnesses (Figure 3). Instead, electronic laboratory-based reporting should become integrated with and complement the existing conventional reporting system to ensure the most complete capture of notifiable disease events.

The findings from this evaluation indicate that direct automatic reporting from a health system is feasible and as complete but more rapid than conventional reporting. An error analysis showed many correctable problems; better control of the use of free text and an ability to retract preliminary reports were key areas for improvement. Standard coding schemes should be used. Health departments need to evaluate electronic surveillance systems before integrating the data into existing reporting systems. CDC and state health departments should collaborate to develop a consensus on the goals for an electronic laboratory-based reporting system intended for public health laboratory-based disease reporting. Once these goals have been determined, guidelines may be created that would assess if the system achieves these desired goals. The methodology in this evaluation may be used by health departments when evaluating other electronic surveillance systems, taking into consideration the different design issues of such systems.

Acknowledgments

Research Studies: Articles should be 2,000 to 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two.

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

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Address for correspondence: Lee H. Harrison, Infectious Diseases Epidemiology Research Unit, University of Pittsburgh, 521 Parran Hall, 130 De Soto Street, Pittsburgh, PA 15261, USA; fax: 412-624-2256; e-mail: lharriso@edc.gsph.pitt.edu

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Infection by *Ralstonia* Species in Cystic Fibrosis Patients: Identification of *R. pickettii* and *R. mannitolilytica* by Polymerase Chain Reaction

Tom Coenye,* Peter Vandamme,† and John J. LiPuma*

The frequency of respiratory tract infections caused by *Ralstonia* species in persons with cystic fibrosis (CF) and the role of these species in CF pulmonary disease are not well documented. In part, this lack of documentation may be attributed to the difficulty in accurately identifying *Ralstonia* species; *R. mannitolilytica* and *R. pickettii* in particular may be misidentified as other closely related species, particularly those of the *Burkholderia cepacia* complex. We used polyphasic analyses to identify 42 *Ralstonia* isolates from sputum cultures from 38 CF patients. Several isolates that could not be identified to the species level may belong to novel *Ralstonia* species. We demonstrated chronic colonization by using genotyping of serial isolates recovered from the same patient. To facilitate identification of *R. mannitolilytica* and *R. pickettii*, we developed 16S ribosomal DNA-based polymerase chain reaction assays that allow sensitive and specific identification of these species.

Cystic fibrosis (CF) is the most frequent hereditary disease in Caucasian populations (1); chronic microbial colonization of the large airways, leading to exacerbations of pulmonary infection, is the major cause of illness and death in CF patients. Typical CF pathogens include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenzae,* and *Burkholderia cepacia* complex; other species, including *Stenotrophomonas maltophilia*, *Alcaligenes* (*Achromobacter*) *xylosoxidans*, *B. gladioli,* and *R. pickettii* have been recovered from sputum cultures of CF patients as well (2,3). Recently, we showed that a number of unusual bacterial species (including several novel species within the α-*Proteobacteria*) are also occasionally isolated from CF patients (4). Infection with mucoid *P. aeruginosa* and members of the *B. cepacia* complex is associated with increased illness and death in CF patients (5–7), but the clinical importance of infection with these other species is less clear.

The genus *Ralstonia* was proposed in 1995 (8). Since its creation, the taxonomy of the genus has expanded to include 11 species, which are *R. pickettii*, *R. solanacearum*, *R. eutropha*, *R. gilardii*, *R. paucula*, *R. basilensis*, *R. oxalatica*, *R. mannitolilytica*, *R. taiwanensis*, *R. campinensis*, and *R. metallidurans* (8–14). *Ralstonia* spp. are isolated from a wide variety of ecologic niches, including plants and soils contaminated with heavy metals. *R. pickettii* has been associated with nosocomial outbreaks caused by contaminated solutions used for patient care and with pseudoepidemics caused by contaminated solutions in the diagnostic laboratory (15–21). Several hospital-associated outbreaks attributed to *R. mannitolilytica*

(formerly known as *R. pickettii* biovar 3 or *P. thomasii*) have been described (12,22,23). *R. paucula* and *R. gilardii* have only sporadically been isolated from human clinical samples, including cerebrospinal fluid, bone marrow, wounds, and the respiratory tract (9,10). A complete assessment of the frequency of human infection due to *Ralstonia* species is confounded by the difficulty in accurate species identification by using standard microbiologic techniques. Indeed, these species are frequently misidentified as *P. fluorescens* or *B. cepacia* complex (12,24–26).

We describe the occurrence of several *Ralstonia* species in the respiratory secretions of CF patients. We also describe the development and evaluation of two polymerase chain reaction (PCR) assays for rapid, accurate identification of *R. pickettii* and *R. mannitolilytica*.

Materials and Methods

Bacterial Strains and Study Population

Since early 1997, the *Burkholderia cepacia* Research Laboratory and Repository (University of Michigan, Ann Arbor, MI) has received more than 4,000 bacterial isolates, collected from CF patients receiving care in 145 CF treatment centers in 130 U. S. cities. Isolates received were tentatively identified by the referring microbiology laboratory as *B. cepacia* complex or a related species or were not identified to the species level. From these isolates, we identified 42 *Ralstonia* isolates obtained from 38 patients who had received care in 19 treatment centers in 18 U. S. cities. The type and reference strains of *Ralstonia*, *Pandoraea*, *Burkholderia*, *Alcaligenes*, and *Bordetella* species have been described (9–14). These strains were obtained from the BCCM/LMG-Bacteria Collection

*University of Michigan Medical School, Ann Arbor, Michigan, USA; and †Ghent University, Ghent, Belgium

(Laboratorium voor Microbiologie, Universiteit Gent, Belgium) or were provided by D. Henry (University of British Columbia, Vancouver, Canada). All isolates were grown aerobically on Mueller-Hinton broth (Becton, Dickinson and Company, Cockeysville, MD) supplemented with 1.8% (wt/vol) agar and incubated at 32°C.

Species Identification

We used a polyphasic approach to identify all isolates, including biochemical tests, 16S ribosomal (r)DNA-based PCR assays and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins. Biochemical tests (determination of oxidase, lysine decarboxylase, and *o*-nitrophenyl-β-D-galactoside activity; growth on *B. cepacia* selective agar; and oxidation-fermentation of sucrose) were performed as described (27). SDS-PAGE of whole-cell proteins was performed as described (9,10), and isolates were identified by comparison with a database containing protein profiles of all *Ralstonia* species. We used 16S rDNA-based PCR assays (28) to determine whether or not isolates belonged to the genera *Burkholderia* or *Ralstonia* or to the *B. cepacia* complex.

Genotyping of Serial Isolate

Multiple isolates from a single patient were genotyped by randomly amplified polymorphic DNA (RAPD) genotyping as described (29). We digitized gel images with a GelDoc2000 gel analyzer (Bio-Rad Laboratories, Hercules, CA) and stored them as tagged image files. After normalization with the molecular weight marker, patterns were analyzed with Molecular Analyst Fingerprinting Plus software (Bio-Rad Laboratories). Similarities between patterns were calculated by using Pearson's product-moment correlation coefficient. We considered isolates to belong to the same genotype if they shared 90% or more similarity.

Development of Primers for Species-Specific PCR Assays

We retrieved 16S rDNA sequences of all *Ralstonia* spp. and representatives of related genera from the GenBank database, using the MegAlign software package (DNASTAR Inc., Madison, WI) to align the sequences. Based on this alignment, we developed primers specific for *R. pickettii* and *R. mannitolilytica*: Rp-F1 (5´-ATGATCTAGCTTGCTAGATTGAT-3´) and Rp-R1 (5´-ACTGATCGTCGCCTTGGTG-3´) (forward and reverse primers for the identification of *R. pickettii*) and Rm-F1 (5´-GGGAAAGCTTGCTTTCCTGCC-3´) and Rm-R1 (5´-TCCGGGTATTAACCAGAGCCAT-3´) (forward and reverse primers for the identification of *R. mannitolilytica*).

Polymerase Chain Reaction

DNA was prepared as described (30). PCR assays were performed in 25-µL reaction mixtures, containing 2 µL DNA solution, 1U *Taq* polymerase (GIBCO Invitrogen Corp., Gaithersburg, MD), 250 mM (each) deoxynucleotide triphosphate (GIBCO Invitrogen Corp.), 1.5 mM MgCl$_2$, 1x PCR buffer

(GIBCO Invitrogen Corp.), and 20 pmol of each oligonucleotide primer. Amplification was carried out with a PTC-100 programmable thermal cycler (Labtrade Inc., Miami, FL). After initial denaturation for 2 min at 94°C, 30 amplification cycles were completed, each consisting of 1 min at 94°C, 1 min at 55°C (for identifying *R. pickettii*) or 57°C (for identifying *R. mannitolilytica*), and 1 min 30 s at 72°C. A final extension of 10 min at 72°C was applied. Negative control PCRs with all reaction mixture components except template DNA were used for every experiment.

Evaluation of the PCR Assays

For evaluating PCR assays, we tested 152 isolates, including 79 *Ralstonia* isolates (both clinical isolates and reference strains) and 73 isolates representing phylogenetically related species that may be found in sputum cultures of CF patients. Isolates tested were as follows: *R. pickettii* (27 isolates), *R. mannitolilytica* (34), *R. gilardii* (4), *R. paucula* (2), *R. taiwanensis* (1), *R. basilensis* (1), *R. eutropha* (1), *R. oxalatica* (1), *R. solanacearum* (1), *R. campinensis* (1), *R. metallidurans* (1), *Ralstonia* sp. (5), *B. cepacia* genomovar I (3), *B. multivorans* (2), *B. cepacia* genomovar III (7), *B. stabilis* (2), *B. vietnamiensis* (2), *B. cepacia* genomovar VI (5), *B. ambifaria* (3), *B. gladioli* (6), *B. fungorum* (1), *Pandoraea apista* (5), *P. norimbergensis* (3), *P. pnomenusa* (2), *P. sputorum* (4), *P. pulmonicola* (2), *Alcaligenes xylosoxidans* (5), *P. aeruginosa* (5), *S. maltophilia* (5), and one isolate each of *A. denitrificans*, *A. piechaudii*, *A. faecalis*, *A. ruhlandii*, *Bordetella avium*, *B. hinzii*, *B. trematum*, *B. bronchiseptica*, *B. pertussis*, *B. parapertussis,* and *B. holmesii*.

Results

Species Identification

Isolates were tentatively identified as belonging to the genus *Ralstonia* if they 1) reacted with primer pair RHG-F/RHG-R (specific for *Burkholderia* and *Ralstonia* spp.) (28), 2) showed no lysine decarboxylase and *o*-nitrophenyl-β-D-galactoside activity, 3) produced no acid from sucrose, and 4) showed oxidase activity. Using these criteria, we identified 42 putative *Ralstonia* sp. isolates. These isolates were further identified to the species level by using SDS-PAGE of whole-cell proteins. Most isolates (25) were identified as *R. mannitolilytica*; 9 were identified as *R. pickettii*. Two isolates were identified as *R. gilardii,* and another as *R. taiwanensis*. Five isolates clearly belonged to the genus *Ralstonia* but could not be classified into one of the known species. Pending further investigations, these isolates were classified as *Ralstonia* sp.

Genotyping of Serial Isolates

We identified two patients (A and B) who were sputum-culture positive for *R. mannitolilytica* and one patient (C) who was culture positive for *R. pickettii* on more than one occasion. The three *R. mannitolilytica* isolates cultured from patient A were recovered over a period of >2 years. RAPD genotyping

indicated that the first isolate clearly differed from the two isolates recovered subsequently; the latter two isolates (recovered 20 months apart) were the same genotype (Figure 1). Similarly, the two *R. mannitolilytica* isolates recovered from patient B (cultured 8 weeks apart) were the same genotype, as were the two *R. pickettii* isolates recovered from patient C (cultured 6 weeks apart) (Figure 1).

Primer Design

Alignment of 16S rRNA gene sequences of *Ralstonia* sp. available in GenBank showed similarity values ≥93.1% and ≥98.2% within the species *R. pickettii* and *R. mannitolilytica*, respectively. Identity of sequences between these two species ranged from 89.9% to 96.8%. Several species-level sequence signatures were detected and were incorporated into the species-specific primers Rp-F1 and Rp-R1 (forward and reverse primer for *R. pickettii*) and Rm-F1 and Rm-R1 (forward and reverse primer for *R. mannitolilytica*). PCR with these primers resulted in the amplification of fragments of 210 bp and 398 bp, respectively (Figure 2). Each of the 152 strains included in this study was examined by PCR with the primer pairs described (Table).

Discussion

The occurrence and clinical role of *Ralstonia* sp. in the respiratory secretions of persons with CF have not been systematically investigated because of the rapidly changing taxonomy of the genus *Ralstonia* and the absence of rapid, reliable methods for species identification. We used a polyphasic approach to identify *Ralstonia* sp. in sputum cultures of CF patients and developed two PCR assays for identifying *R. pickettii* and *R. mannitolilytica*.

Previous reports describing the bacterial flora of the respiratory tract of CF patients have focused mainly on *P. aeruginosa* and *B. cepacia* complex organisms (3,5,31); reports describing the presence of *Ralstonia* species in sputum cul-

tures of CF patients are scarce and often anecdotal. In a prospective study, Burns et al. (2) isolated *R. pickettii* from only 2 of 559 patients. More recently, we have shown that other *Ralstonia* species, including *R. mannitolilytica*, *R. taiwanensis*, and *R. gilardii,* can also be isolated from the respiratory secretions of CF patients (4). In this study, we identified *Ralstonia* species recovered from sputum cultures of 38 CF patients. Collectively, these data indicate that the prevalence of *Ralstonia* sp. in the CF population is rather low. However, because we did not specifically survey all referring laboratories for all *Ralstonia* species that may have been recovered from CF specimens, we were not able to define a more precise prevalence of *Ralstonia* sp. in the CF population.

Our data do not provide evidence for patient-to-patient spread of *Ralstonia* sp. because no clustering of cases occurred within centers or geographic regions (data not shown). However, we were able to document persistent colonization with *Ralstonia* species in three patients. Patient A's infection is particularly interesting. In this patient, an initial *R. mannitolilytica* strain was apparently replaced with another strain, which then persisted for >20 months. However, the bacterial and host factors involved in infection by more than one *R. mannitolilytica* strain or with chronic colonization remain to be defined.

Five *Ralstonia* isolates could not be identified to the species level. 16S rDNA PCR and SDS-PAGE of whole-cell proteins clearly indicated that these isolates belong to the genus *Ralstonia*, suggesting that they may represent novel *Ralstonia* sp. Further polyphasic taxonomic studies are needed to clarify their status. The finding of *R. mannitolilytica*, *R. gilardii*, *R. taiwanensis,* and possible novel *Ralstonia* species in respiratory secretions of CF patients suggests that these organisms may be emerging human pathogens and again highlights the fact that the bacterial biodiversity in the respiratory tract of CF patients has thus far been underestimated (4).

Of the 25 *R. mannitolilytica* strains identified in this study, 9 were initially identified by the referring laboratory as *R. pickettii*, 8 as *B. cepacia* complex, 6 as *Burkholderia* sp., 1 as *B. gladioli*, and 1 as *P. fluorescens*. Of the 9 *R. pickettii* strains identified, 3 were identified by the referring laboratory as *R. pickettii*, 2 as *Burkholderia* sp., 1 as *Pseudomonas* sp., 1 as *B. cepacia* complex, and 2 isolates as unidentified. The *R. gilardii* and *R. taiwanensis* isolates were received as *B. cepacia* complex and *S. maltophilia*, respectively. Most (81%) of these isolates were capable of growth on *B. cepacia* selective agar. These observations reiterate that identification of these species is not straightforward and that their misidentification as other CF pathogens, such as *B. cepacia* complex, is not uncommon. Such misidentification has an important impact on infection control in CF since the efficiency of these measures depends on accurate identification of the microorganisms involved. Infection-control policies, particularly those recommended to prevent interpatient spread of *B. cepacia* complex, have a tremendous impact on the quality of life of CF patients (6,7). To enhance accurate identification of CF pathogens, several PCR assays have been developed recently (28,30,32–35). We
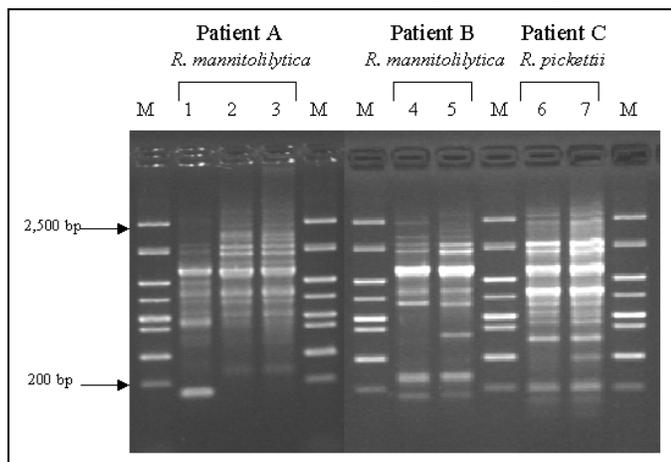
Figure 1. Randomly amplified polymorphic DNA analysis of serial isolates from three patients. M: molecular weight marker; lanes 1, 2, and 3: serial *Ralstonia mannitolilytica* isolates from patient A in chronological order; lanes 4 and 5: serial *R. mannitolilytica* isolates from patient B in chronological order; and lanes 6 and 7: serial *R. pickettii* isolates from patient C in chronological order.
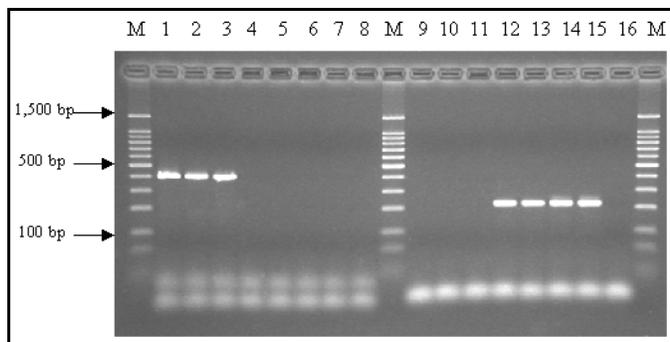
Figure 2. Polymerase chain reaction analysis of *Ralstonia* strains with primer pairs Rm-F1/Rm-R1 (lanes 1–8) and Rp-F1/Rp-R1 (lanes 9–16). M: 100-bp DNA ladder; lanes 1, 2, 3, 9, 10, and 11: *R. mannitolilytica*; lanes 4, 5, 6, 7, 12, 13, 14, and 15: *R. pickettii*; and lanes 8 and 16: *R. gilardii*.

sought to design similar PCR tests to allow the identification of *R. pickettii* and *R. mannitolilytica* based on species-level signature sequences in the 16S rRNA gene. By comparing available *R. pickettii* and *R. mannitolilytica* 16S rRNA gene sequences with sequences from other *Ralstonia* species and representatives of the phylogenetically closely related genera *Burkholderia* and *Pandoraea*, we identified several regions that showed sufficient diversity to allow the design of primer pairs Rp-F1/Rp-R1 and Rm-F1/Rm-R1, permitting the sensitive and specific identification of *R. pickettii* and *R. mannitolilytica*, respectively (Table).

The results of our study indicate that a number of *Ralstonia* species can be isolated from sputum cultures of CF patients. The correct identification of these species presents a challenge for diagnostic microbiology laboratories. Our study supports the use of genotypic methods to augment routine phenotypic evaluation. The combined use of the two PCR assays described will allow the identification of most *Ralstonia* species encountered in sputum cultures of CF patients. Most importantly, the use of these assays will substantially reduce the misidentification of *R. pickettii* and *R. mannitolilytica* as *B. cepacia* complex. These tests will be a valuable adjunct in the

evaluation of CF sputum culture isolates and will allow more precise study of the prevalence and natural history of human infection by these emerging pathogens.

Dr. Coenye is a postdoctoral research fellow in the Department of Pediatrics and Communicable Diseases at the University of Michigan. His major research interests are the identification, biodiversity, and molecular epidemiology of bacteria associated with pulmonary infections in cystic fibrosis patients.

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Table. Sensitivity and specificity of polymerase chain reaction (PCR) assays for the identification of *Ralstonia mannitolilytica* and *R. pickettii*

Primer pair and species tested	Sensitivity (%)	Specificity (%)	No. of strains	
			Positive	Negative
Rp-F1/Rp-R1				
R. pickettii (n=27)	89	99	24	3[a]
All others (n=125)			1[b]	124
Rm-F1/Rm-R1				
R. mannitolilytica (n=34)	100	99	34	0
All others (n=118)			1[c]	117

[a]Of the three *R. pickettii* isolates that gave a false-negative reaction in this PCR test, two were identified as *R. pickettii* by the referring laboratory, while one was received unidentified.
[b]One unidentified *Ralstonia* sp. isolate cross-reacted with this primer pair.
[c]One *R. pickettii* isolate cross-reacted with this primer pair.

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Address for correspondence: Tom Coenye, Department of Pediatrics and Communicable Diseases, 8301 MSRB III, Box 0646, 1150 W. Medical Center Dr., Ann Arbor, Michigan 48109-0646, USA; fax: 734-615-4770; e-mail: tcoenye@umich.edu

Temporal Changes in Prevalence of Antimicrobial Resistance in 23 U.S. Hospitals

Scott K. Fridkin,* Holly A. Hill,† Nataliya V. Volkova,† Jonathan R. Edwards,*
Rachel M. Lawton,* Robert P. Gaynes,* John E. McGowan, Jr.,† and the Intensive Care
Antimicrobial Resistance Epidemiology (ICARE) Project Hospitals

Antimicrobial resistance is increasing in nearly all health-care–associated pathogens. We examined changes in resistance prevalence during 1996–1999 in 23 hospitals by using two statistical methods. When the traditional chi-square test of pooled mean resistance prevalence was used, most organisms appear to have increased in prevalence. However, when a more conservative test that accounts for changes within individual hospitals was used, significant increases in prevalence of resistance were consistently observed only for oxacillin-resistant *Staphylococcus aureus*, ciprofloxacin-resistant *Pseudomonas aeruginosa*, and ciprofloxacin- or ofloxacin-resistant *Escherichia coli*. These increases were significant only in isolates from patients outside intensive-care units (ICU). The increases seen are of concern; differences in factors present outside ICUs, such as excessive quinolone use or inadequate infection-control practices, may explain the observed trends.

The increasing prevalence of antimicrobial-resistant organisms, a major public health problem, is of particular concern for hospitals (1,2). However, resistance data aggregated from many hospitals document changes over time but often do not evaluate the consistency of these changes in all the hospitals (3–5). Several statistical tests can be used to evaluate changes in antimicrobial-resistance prevalence; chi-square is commonly used but does not account for consistency of trends in all hospitals. Thus, national or international evaluations based on observed changes in resistance patterns in isolates pooled from all sites can misrepresent the overall trend if a few of the sites report outlier data, as had been observed with data from the National Nosocomial Infections Surveillance system (6). A second difficulty with interpreting data for U.S. trends of antimicrobial resistance in health-care settings is inherent in the diversity of populations served by the facilities.

Monitoring resistance patterns by location within the hospital (e.g., intensive-care units [ICUs], non-ICU inpatient areas, and outpatient areas) can demonstrate substantial changes that would be obscured if hospitalwide data were aggregated into national trends. To determine consistency of changes in antimicrobial-resistance patterns over time in a national monitoring project, we used two statistical methods to evaluate national antimicrobial-resistance data over a 4-year period, as well as assess consistency within hospitals.

Methods

For this study, we monitored changes in antimicrobial resistance in different hospital areas during two periods (1996–1997 and 1998–1999) in facilities participating in Project ICARE (Intensive Care Antimicrobial Resistance Epi-

demiology), a joint project of the Hospital Infections Program (now the Division of Healthcare Quality Promotion) of the Centers for Disease Control and Prevention (CDC) and the Rollins School of Public Health of Emory University. Hospitals participating in the ICU surveillance component of the National Nosocomial Infections Surveillance (NNIS) system were invited to participate in the second (January 1996 through December 1997) and third (April 1998 through July 1999) phases of Project ICARE. Twenty-three U.S. hospitals reported acceptable data for both time periods. The surveillance methods and definitions of the NNIS system and Project ICARE have been described (7,8).

Each month, hospitals reported the antimicrobial-susceptibility results of isolates recovered from clinical specimens from patients served by the clinical microbiology laboratory. For study isolates, susceptibility results were reported from all clinical specimens, whether associated with hospital- or community-acquired infection or colonization. Duplicate isolates were excluded; these were defined as isolates of the same organism with the same antimicrobial-resistance pattern recovered from the same patient during a calendar month, regardless of the site of isolation (e.g., blood, sputum, urine, wound). In addition, isolates obtained as part of infection-control surveillance were excluded. When these "surveillance" isolates are excluded, the resistance prevalence (i.e., percent resistant) more closely reflects data routinely aggregated as part of the laboratories' cumulative susceptibility reports (i.e., cumulative antibiograms). The validity of the susceptibility data has been assessed, and participating laboratories were evaluated as performing reliably. This assessment was done through a proficiency testing program at these laboratories, as well as confirmatory testing of selected isolates (9).

Susceptibility results (MIC and zone diameters) were interpreted according to criteria from the National Committee

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and †Emory University, Atlanta, Georgia, USA

for Clinical Laboratory Standards (NCCLS) (10–12). The sentinel organisms considered in the analysis, which represented frequently encountered resistance problems in U.S. hospitals, were oxacillin-resistant coagulase-negative staphylococci, oxacillin-resistant *Staphylococcus aureus* (ORSA), vancomycin-resistant enterococci (VRE), third-generation cephalosporin-resistant *Escherichia coli*, third-generation cephalosporin-resistant *Enterobacter* species, ceftazidime-resistant *Pseudomonas aeruginosa*, ciprofloxacin-resistant *P. aeruginosa*, ciprofloxacin-resistant *E. coli* (for *E. coli*, defined as resistance to either ofloxacin or ciprofloxacin), and third-generation cephalosporin-resistant *Klebsiella pneumoniae*.

These data were aggregated for each month and stratified by hospital area and time period. To determine temporal trends, we compared all data reported from isolates tested during 1996–1997 (period 1) with all data reported from isolates tested during 1998–1999 (period 2). Data were reported for each hospital area, including each separate ICU (units that provide intensive observation, diagnosis, and therapeutic procedures for critically ill patients); as a pooled total for a given hospital's non-ICU inpatient areas (areas other than ICUs where the patient stays at least one night in the hospital); and as a pooled total for each hospital's outpatient areas (urgent care or emergency wards and units that perform same-day surgery or simple diagnostic procedures and therapy, such as chemotherapy, hemodialysis, or cardiac catheterization). Pooled rates were calculated for prevalence of resistance (e.g., percent VRE = proportion of enterococci tested that were resistant to vancomycin) at each hospital. If <10 isolates were tested for antimicrobial susceptibility from a specific hospital area during the study period, the prevalence rate was considered to be of low accuracy, and that hospital area was excluded from further analysis.

To assess the overall magnitude of resistance for each sentinel organism, we calculated an overall (i.e., weighted mean) pooled mean prevalence, combining data from all hospitals, by hospital area and time period. Changes in resistance prevalence over time within each hospital area were assessed by chi-square tests. In addition, each hospital's change in resistance prevalence over time for each sentinel organism was determined by subtracting the period 2 rate from the period 1 rate. Since the changes in resistance rates for most organisms did not follow a normal distribution, a nonparametric test was used to assess the statistical significance of the temporal changes. The Wilcoxon signed-rank test was chosen for the analysis to take into account the variability of resistance patterns in individual hospitals while minimizing the impact of hospitals with extreme (outlying) values of temporal changes in resistance. The signed-rank test is used to assess the null hypothesis that the population median of the differences in paired observations is equal to zero (13). Since the focus is on medians rather than means, extreme values are less likely to influence the outcome of this test, unlike a t test of the pooled mean values. P values <0.05 were considered significant.

Results

Of 61 hospitals reporting some data to Project ICARE in either period, 23 (38%) reported at least 6 months of data during both periods and were included in this analysis. Twenty-one (91%) were general hospitals, and 2 (9%) were Veterans Administration hospitals. Fifteen (65%) were affiliated with a medical school. The mean size of participating hospitals was 440 beds (median 356, range 147–1,022); 13 (56%) were in the Atlantic Region, 6 (26%) in the Central Region, 2 (9%) in the New England Region, and 2 (9%) in the Pacific Region. Study hospitals (n=23) did not differ significantly in these characteristics from the ICARE hospitals that were excluded from analysis (n=38) because they submitted data for only one of the two time periods.

The overall pooled mean prevalence of resistance from period 1 to period 2 appeared to have changed for most of the sentinel organisms. The changes were statistically significant when compared by a chi-square test of the pooled means, by time period, for five sentinel organisms in the ICU areas, five in the non-ICU areas, and four in the outpatient areas (Table, footnote). However, when the temporal change in prevalence was evaluated by comparing the median difference in prevalence between period 1 and period 2, no organism demonstrated a significant temporal change in prevalence in the ICUs (Table). In addition, temporal changes remained significant for only three of the sentinel organisms in the non-ICU inpatient area and four in the outpatient area. In non-ICU inpatient areas, significant increases in median resistance rates were noted for ORSA (8.2%), ciprofloxacin-resistant *P. aeruginosa* (3.3%)*,* and ciprofloxacin-resistant *E. coli* (0.6%) (Table). In outpatient areas, significant increases in median resistance rates were evident for ORSA (2.4%), VRE (0.6%), ciprofloxacin-resistant *E. coli* (1.0%), and ciprofloxacin-resistant *P. aeruginosa* (5.0%) (Table). No significant change in resistance prevalence was observed for oxacillin-resistant coagulase-negative staphylococci, third-generation cephalosporin-resistant *E. coli*, third-generation cephalosporin-resistant *Enterobacter* species, or ceftazidime-resistant *P. aeruginosa* in ICU, non-ICU inpatient, or outpatient areas.

Discussion

These data, which demonstrate a high level of antimicrobial resistance in organisms commonly associated with hospital-acquired infections, are consistent with other reports (3–5,14). However, in this analysis of data from 23 hospitals for 1996–1999, we demonstrate that antimicrobial resistance in the study hospitals has increased consistently for only a few of the sentinel organisms measured. Significant increases were limited to ORSA, ciprofloxacin-resistant *P. aeruginosa*, and ciprofloxacin- or ofloxacin-resistant *E. coli*. Furthermore, these increases were significant only for isolates obtained from non-ICU unit areas. If the traditional chi-square test, which uses the pooled mean prevalence rate, is used to determine the level of significance, significant increases appear to have occurred in most of the organisms studied and throughout the

Table. Weighted pooled mean prevalence and temporal differences of antimicrobial resistance for sentinel organisms, 1996–1999, Project ICARE hospitals

Antimicrobial-Resistant Pathogen	Weighted pooled mean resistance rate (%)		Median difference (%) in resistance rates[a]	N	p value[b]
	1996–1997	1998–1999			
Intensive-care unit areas					
Oxacillin-resistant CNS	76.0	73.6	-0.01	20	0.8
Oxacillin-resistant *Staphylococcus aureus*	30.9	35.6[c]	1.83	22	0.4
Vancomycin-resistant enterococcus	15.5	15.0	-1.81	20	0.9
Cef3-resistant *Escherichia coli*	0.57	2.2[c]	0.00	20	0.3
Cef3-resistant *Enterobacter* spp.	25.2	25.0	-2.08	17	0.4
Ceftazidime-resistant *P. aeruginosa*	8.3	7.8	0.37	21	0.9
Ciprofloxacin-resistant *P. aeruginosa*	17.7	24.4[c]	0.63	22	0.2
Ciprofloxacin-resistant *E. coli*	0.9	2.0[c]	0.00	20	1.0
Cef3-resistant *Klebsiella pneumoniae*	2.4	8.4[c]	0.00	18	0.3
Non-intensive–care unit inpatient areas					
Oxacillin-resistant CNS	62.6	63.6	0.41	20	0.6
Oxacillin-resistant *Staphylococcus aureus*	30.2	34.4[c]	8.20	22	0.008
Vancomycin-resistant enterococcus	13.9	11.3	0.93	22	0.4
Cef3-resistant *Escherichia coli*	0.69	0.53	0.00	20	0.9
Cef3-resistant *Enterobacter* spp.	22.1	20.5	-5.90	21	0.4
Ceftazidime-resistant *P. aeruginosa*	5.8	5.9	0.00	21	0.9
Ciprofloxacin-resistant *P. aeruginosa*	17.2	23.9[c]	3.30	22	0.02
Ciprofloxacin-resistant *E. coli*	1.4	2.5[c]	0.57	22	0.008
Cef3-resistant *K. pneumoniae*	3.6	4.9[c]	0.06	20	0.1
Outpatient/urgent/emergent care patients					
Oxacillin-resistant CNS	45.2	43.6	11.50	21	0.4
Oxacillin-resistant *Staphylococcus aureus*	18.0	22.6[c]	2.40	22	0.009
Vancomycin-resistant enterococcus	2.1	4.8[c]	0.61	21	0.02
Cef3-resistant *Escherichia coli*	0.16	0.23	0.00	22	0.7
Cef3-resistant *Enterobacter* spp.	10.0	9.2	-0.77	21	0.6
Ceftazidime-resistant *P. aeruginosa*	3.8	3.6	0.16	21	0.7
Ciprofloxacin-resistant *P. aeruginosa*	20.0	24.6[c]	5.00	21	0.02
Ciprofloxacin-resistant *E. coli*	0.61	1.4[c]	1.00	22	<0.001
Cef3-resistant *K. pneumoniae*	1.1	1.5	0.00	20	0.5

[a]Median of the differences in resistance prevalence from period 1 (1996–1997) to period 2 (1998–1999) observed in the (N) hospitals or units reporting resistance information on ≥10 isolates for each of the time periods. CNS, coagulase-negative Staphylococcus; Cef3, ceftazidime, cefotaxime, or ceftriaxone; for *E. coli,* ciprofloxacin resistance is resistance to either ciprofloxacin or ofloxacin.
[b]p value by Wilcoxon signed-rank test of the differences at N hospitals or units.
[c]p<0.05 by chi-square test of pooled mean resistance rates between time periods.

hospital. However, these overall changes in prevalence often were influenced by weighting of the pooled mean by a few hospitals reporting larger numbers of isolates or very large increases in antimicrobial-resistance prevalence. The data from these influential hospitals were not representative of what was observed in most of the hospitals. Thus, the more conservative statistical test used, the Wilcoxon signed-rank test, identified those hospital areas and sentinel organisms where the temporal change was more representative of all the hospitals. By using the more conservative assessment of median differences between time periods, we were able to present a more valid scenario of observations across most of our study hospitals. The paired t test, which tests whether the mean difference in resistance prevalence is equal to zero, is also a viable alternative for analysis of data such as these, provided that sample sizes are large enough to justify the

assumption that the differences are normally distributed. If uncertainty exists about the normal distribution, the Wilcoxon signed-rank test is a good choice, since it performs almost as well as the t test when the data are normally distributed.

Although the prevalence of ORSA has not increased in the ICUs of these hospitals, the increase in prevalence of ORSA outside ICUs is very concerning. *S. aureus* is commonly seen with central line-associated bloodstream or surgical site infection (15). The median increase of 2.4% in isolates from the outpatient areas is approximately a 10% increase over the baseline prevalence observed in the first time period (i.e., 20% ORSA). Although these isolates are mostly from emergency room patients who likely have had recent exposures to health-care settings, this prevalence rate is comparable with the rate of 20%–23% observed in hospitalized patients in the early 1990s (16). With more frequent reports of community-onset ORSA infections (17–19), we expect this prevalence rate to continue to increase unless adequate prevention measures are identified and implemented.

Gram-negative bacilli are frequently associated with hospital-acquired infections, particularly ventilator-associated pneumonia and catheter-associated urinary tract infections (15,20). Although antimicrobial resistance in these organisms to third-generation cephalosporins is of great clinical concern (4,21,22), no consistent increases occurred in prevalence of third-generation cephalosporin resistance in *E. coli*, *Enterobacter* spp., *K. pneumoniae*, or *P. aeruginosa*. This finding does not imply that some hospitals did not experience significant increases, but rather that changes over time were not consistent between facilities in all hospital areas. This observation may reflect successful infection-control strategies in study hospitals, but further study is needed to validate this conjecture. For *K. pneumoniae* or *E. coli*, these data suggest that ESBL-producing *K. pneumoniae* or *E. coli* remains a focal problem.

The data are strikingly different for ciprofloxacin resistance in *P. aeruginosa* and *E. coli*. With these organisms, resistance from non-ICU patient isolates and outpatient isolates increased across all hospitals, but resistance in the ICU patient isolates did not increase significantly. Contributing factors may include the large amounts of quinolones used by patients outside the ICU or the development of ciprofloxacin resistance in *P. aeruginosa* unrelated to the ICU setting (23).

No consistent increases in resistance were observed in ICU isolates for any of the study organisms, which may reflect successful infection-control programs in the ICUs at these study hospitals. However, this finding also might reflect a variation in the evolution of antimicrobial resistance at these hospitals. For example, the prevalence of antimicrobial resistance increased first in the ICUs, and factors similar to those in the ICUs have moved into the non-ICU areas, resulting in increases in these areas during the second half of the study.

One major limitation of this study is the small size of this national sample. With only 23 hospitals reporting sufficient data in each study period, inferences from these data about the direction of antimicrobial resistance in the United States overall must be made with caution. Although these hospitals are representative of all NNIS hospitals, hospitals in the Mid- and South-Atlantic regions are overrepresented (24). However, statistically significant trends of increasing resistance for ORSA and ciprofloxacin-resistant *P. aeruginosa* or *E. coli,* found using a conservative test for significance, suggest that these changes are consistent in all study hospitals. This finding may indicate that these resistant organisms represent problems faced by most U.S. hospitals.

Another limitation is lack of confirmation of the clinical relevance of the organisms evaluated in this study, which represent organisms associated both with colonization and infection. However, we minimized inclusion of colonizing organisms by eliminating duplicate reports. In addition, in a separate analysis of these surveillance data, we have demonstrated that the cumulative susceptibility reports generated from these data are comparable with those for organisms reported to be associated with definitive hospital-acquired infection (25). Therefore, we believe the data in this study are representative of the susceptibilities of the organisms associated with hospital-acquired infections.

These data suggest that monitoring antimicrobial resistance by hospital area can identify national trends in resistance prevalence affecting only certain hospital areas. Increases are also widespread in study hospitals in patients outside the ICU. Attention should be paid to identifying novel measures for curbing increases in antimicrobial resistance outside ICUs and to assessing why current measures are failing.

Aggregated susceptibility data, such as those presented here, may be easily obtained as part of local or regional surveillance efforts. Written guidelines for producing cumulative susceptibility reports from hospital-based surveillance efforts have been created by the National Committee for Clinical Laboratory Standards (26). Public health authorities can use such data produced by standard specifications to assess trends in prevalence of antimicrobial-resistant organisms associated with health-care delivery. However, analysis of temporal trends should include assessing consistency of changes in the facilities under surveillance by using appropriate statistical tests.

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Dr. Fridkin is a medical epidemiologist in the Division of Healthcare Quality Promotion (formerly the Hospital Infections Program), National Center for Infectious Diseases, Centers for Disease Control and Prevention. He works primarily on antimicrobial-resistance initiatives, including surveillance of antimicrobial resistance in health care, identification and control of antimicrobial-resistant infections, and outbreak investigations involving new and emerging antimicrobial-resistant pathogens in health-care settings.

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Address for correspondence: Scott K. Fridkin, Division of Healthcare Quality Promotion, Mailstop A35, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30333, USA; fax: 404-639-6458; e-mail: skf0@cdc.gov

Research is the act of going up alleys to see if they are blind

— Plutarch

Monitoring Antimicrobial Use and Resistance: Comparison with a National Benchmark on Reducing Vancomycin Use and Vancomycin-Resistant Enterococci

Scott K. Fridkin,* Rachel Lawton,* Jonathan R. Edwards,* Fred C. Tenover,*
John E. McGowan, Jr.,† Robert P. Gaynes,* the Intensive Care Antimicrobial
Resistance Epidemiology (ICARE) Project, and the National Nosocomial Infections
Surveillance (NNIS) System Hospitals

To determine if local monitoring data on vancomycin use directed quality improvement and decreased vancomycin use or vancomycin-resistant enterococci (VRE), we analyzed data from 50 intensive-care units (ICUs) at 20 U.S. hospitals reporting data on antimicrobial-resistant organisms and antimicrobial agent use. We compared local data with national benchmark data (aggregated from all study hospitals). After data were adjusted for changes in prevalence of methicillin-resistant *Staphylococcus aureus*, changes in specific prescriber practice at ICUs were associated with significant decreases in vancomycin use (mean decrease -48 defined daily doses per 1,000 patient days, p<0.001). These ICUs also reported significant decreases in VRE prevalence compared with those not using unit-specific changes in practice (mean decrease of 7.5% compared with mean increase of 5.7%, p<0.001). In this study, practice changes focused towards specific ICUs were associated with decreases in ICU vancomycin use and VRE prevalence.

The emerging problem of antimicrobial resistance in bacterial pathogens is very complex (1,2). However, one common theme is that antecedent antimicrobial exposure exerts selective pressure favoring the emergence of resistance (2). Appropriate antimicrobial use is an integral component of any program to slow the emergence and spread of antimicrobial-resistant microorganisms in the health-care setting (1,3). The optimal methods to reduce inappropriate or excessive antimicrobial use will differ by institution. Although many possible interventions have been proposed (4), deciding which one is the most effective in a particular setting can be difficult. Despite guidelines from governmental and professional groups (3,5–7), many hospitals have yet to institute any antimicrobial use policies or programs to improve antimicrobial agent prescribing (8).

The Infectious Disease Society of America and the Society for Healthcare Epidemiology of America Joint Committee on the Prevention of Antimicrobial Resistance recently published guidelines for the prevention of antimicrobial resistance in hospitals (3). Two of the six broad recommendations were to establish a system for monitoring bacterial resistance and antibiotic use and to establish practice guidelines and other institutional policies to control the use of antibiotics and respond to

data from the monitoring system. Responding to data from a local monitoring system, especially in the context of an external benchmark, has been a successful way to create practice changes to improve the quality of patient care (9,10). Efforts have been made to improve outcomes for hospitalized patients; success has been demonstrated with surgical site infections and more recently, with catheter-related bloodstream infections (11,12). In both examples, local infection rates are compared with those of a sample that serves as an external benchmark. Valid benchmarks for comparing antimicrobial use have not been well established (13). One example of hospitals establishing a monitoring and benchmarking system is Project Intensive Care Antimicrobial Resistance Epidemiology (ICARE), a collaborative study between the Hospital Infections Program (now the Division of Healthcare Quality Promotion) at the Centers for Disease Control and Prevention (CDC) and the Rollins School of Public Health of Emory University. During this 4-year study, a subset of hospitals participating in the National Nosocomial Infections Surveillance (NNIS) system monitored antimicrobial use. We present data from Project ICARE that demonstrate how hospitals used local data and national benchmark data to effect practice changes resulting in reduced vancomycin use and prevalence of vancomycin-resistant enterococci (VRE) in intensive-care units (ICUs).

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and †Emory University, Atlanta, Georgia, USA

Methods

Setting

Hospitals that participate in the ICU surveillance component of the NNIS system were invited to participate in the second (January 1996 through December 1997) and third (April 1998 through July 1999) phases of Project ICARE; 55 ICUs from 21 hospitals reported the required data to both the second and third phase of Project ICARE. The surveillance methods and definitions of the NNIS system (14,15) and Project ICARE (13) have been previously described. As participants in the NNIS system, ICUs had been previously categorized by the types of patients served: coronary (CCU), medical (MICU), general surgical (SICU), cardiothoracic, combined medical-surgical (<80% of patients can be classified into a single ICU patient type), neurosurgical, respiratory, trauma, burn, or other.

Data Collection

Participating hospitals reported the grams of select antimicrobial agents administered to patients and the antimicrobial susceptibility results of isolates recovered from clinical specimens from hospitalized patients each month. Microbiologic data were aggregated for each ICU separately, all non-ICU inpatient wards combined, and all outpatient areas combined (e.g., units that perform same-day surgery, simple diagnostic procedures or therapy, urgent care, or emergency care). Pharmacy data were reported for the same hospital strata, except for outpatient areas for which pharmacy data were not available. Amounts of antimicrobial drugs reported were standardized by conversion to defined daily doses; for parenteral vancomycin, one daily dose was defined as 2 g. This analysis includes both parenteral and oral (2% of total vancomycin use) vancomycin.

The microbiology laboratory reported antimicrobial susceptibility results for all enterococci and *Staphylococcus aureus* isolates recovered from all clinical specimens, whether associated with hospital- or community-acquired infection or colonization. Duplicate isolates were excluded: these were defined as isolates of the same organism with the same antimicrobial resistance pattern recovered from the same patient, regardless of the site of isolation (e.g., blood, sputum, urine, or wound), during the same calendar month. Susceptibility reports from isolates obtained as part of infection-control surveillance were excluded. When excluding these surveillance isolates, VRE or methicillin-resistant *S. aureus* (MRSA) prevalence more closely reflects data routinely aggregated as part of the cumulative susceptibility report (i.e., cumulative antibiogram). The validity of the susceptibility data submitted by participating hospitals for VRE and MRSA has previously been confirmed through a proficiency testing program at these laboratories and by confirmatory testing at the ICARE reference laboratory of up to 20 VRE and 20 MRSA isolates from these hospitals (13,16). Enterococci were considered vancomycin resistant if the MIC of vancomycin was 32 µg/mL or if

the zone diameter by disk diffusion was 14 mm. *S. aureus* were considered oxacillin (methicillin) resistant if the MIC of oxacillin was 4 µg/mL or if the zone diameter by disk diffusion was 10 mm (17).

Feedback Data

In October 1997, a report of local monitoring data for each hospital area compared to the national benchmark (i.e., aggregate summary data from all 41 Phase 2 ICARE hospitals, including 113 ICUs) was disseminated to each participating hospital (18). The aggregate benchmark data included numeric presentation of pooled means, medians, and key percentile distributions of the prevalence of selected antimicrobial-resistant organisms, stratified by ICU areas combined, non-ICU–inpatient areas combined, and outpatient areas combined (18). In addition, the data for antimicrobial agent use were stratified by the specific type of ICU (e.g., general-surgical separate from cardiothoracic ICU, non-ICU–inpatient areas combined, and outpatient areas combined) (18). Each individual hospital's report included raw data and pooled means of the same target rates for each hospital area. Stratification of use and resistance prevalence by different hospital areas, as described, provided a valid benchmark by which hospitals were able to determine how their usage and resistance prevalence compared with the aggregate, when the data were adjusted for different patient populations in these different hospital areas.

To ascertain how the hospital infection-control staff used the feedback report, they were surveyed in September 1999. Information was collected on the use of the feedback report, recognition of problem pathogens or excessive use of specific antimicrobial agents, and specific practice changes. Questions were open-ended to include any change in infection-control practice (including hygienic practices, barrier precautions, and antimicrobial control practices) from baseline practice (i.e., during pre-intervention period), rather than a description of specific practices already in use. The infection-control practitioner overseeing the surveillance activities responded to the survey, with input from the infection-control committee, based on recollection or meeting minutes.

Data Analysis

For this analysis, monthly data from each ICU were pooled for the entire study period and for each period of the study (i.e., pre-intervention and postintervention) by each ICU (data from non-ICU–inpatient areas and outpatient areas are not shown because of low statistical power). Pooled rates were calculated for prevalence of VRE (percentage), MRSA (percentage), and vancomycin use (defined daily doses/1,000 patient-days). For example, the pooled mean rate of vancomycin use was calculated for each ICU by dividing the total number of defined daily doses by the total number of patient-days reported over the study period by that ICU, multiplied by 1,000, and thus expressed as defined daily doses per 1,000 patient-days. If <10 *S. aureus* or enterococci isolates were tested for antimicrobial susceptibility from a specific ICU

during the study period, that ICU was excluded from further analysis.

Data were analyzed by SAS Release 6.12 Software (SAS Institute Inc., Cary, NC). To assess the change in ICU-specific prevalence of MRSA, VRE, and vancomycin use, the pre-intervention rate was subtracted from the postintervention rate (i.e., difference in rates). Differences in the percent VRE and vancomycin use rate were evaluated by the paired t-test and further compared by type of practice change by a paired t-test. Frequency of MRSA in a hospital has been shown to be independently associated with rates of vancomycin use (19). We used linear regression modeling to determine which types of practice changes were independently associated with changes in vancomycin use in ICUs, after the data were adjusted for each ICU and changes in MRSA prevalence by the GLM procedure (SAS Institute Inc.). Detection of potential influential data points and their influence on main effect factors were also assessed in the modeling process. All reported p values are two-tailed. Analyses were repeated by using the relative change of each parameter rather than difference in rates.

Results

Description of Sites

During the study period, 21 hospitals representing 55 ICUs followed the surveillance protocol and reported at least 6 months of data by the time of the intervention and a median of 32 months (range 18–45) of data during the study period. Twenty (95%) hospitals completed the intervention survey representing the 50 ICUs included in this analysis. These hospitals were from 18 states and had a median hospital bed size of 351 (range 147–1,022); 13 (65%) reported an affiliation with a teaching institution (i.e., major teaching centers), and 2 (10%) were Veterans Affairs Medical Centers. The ICUs included 14 combined medical-surgical ICUs, 7 cardiac care units, 7 MICUs, 8 general SICUs, 6 cardiothoracic ICUs, 4 neurosurgical ICUs, 3 pediatric ICUs, and 1 burn ICU.

Use of Local Data Compared with Benchmark

Infection-control teams at all hospitals disseminated the benchmark data to a variety of hospital committees and personnel (e.g., pharmacy and therapeutics, ICU personnel). This feedback was usually in the form of a committee report or memo. In addition to reporting, 12 (60%) hospitals changed prescribing of vancomycin (i.e., prescriber practice change). Eight (40%) hospitals reported at least one prescriber practice change (many hospitals combined several hospitalwide changes in prescriber practice) that was applied hospitalwide; these changes encompassed 22 ICUs (Table). The hospital-wide changes included evaluating periodic drug use (19 ICUs), redistributing guidelines on appropriate uses of vancomycin (20 ICUs) by newsletter or mail (9 ICUs), and requiring prior approval for use of vancomycin (3 ICUs). In contrast, four hospitals reported focused (i.e., ICU-specific) practice changes in 11 ICUs; these included ICU-specific education in-service

sessions on appropriate vancomycin use (8 ICUs) and removing vancomycin as routine prophylaxis for cardiac surgery (2 ICUs). Both practice changes were reported in one ICU (Table).

Vancomycin Use

In the 50 study ICUs, the rates of vancomycin use during the pre-intervention period (Figure 1, plotted circles) were similar in range to the 113 ICARE Phase 2 ICUs contributing data to the national aggregate benchmark report (Figure 1, box plots). The overall (pooled mean) ICU-specific use of vancomycin in the 50 ICUs at the 20 study hospitals after the intervention was 89.1 defined daily doses per 1,000 patient-days, a 2.8% increase over the pre-intervention rate of use (86.6 defined daily doses per 1,000 patient-days). Despite this increase in aggregate usage among all ICUs, most ICUs reported lower rates of vancomycin use after the intervention compared with the pre-intervention rates. The median difference was -3 defined daily doses per 1,000 patient days (range -138 to +196), but this difference was not statistically significant.

Differences in the rate of vancomycin use varied substantially by the type of practice change. ICUs in which unit-specific programs were implemented used significantly lower rates of vancomycin in the postintervention period than in the pre-intervention period (Table), including both ICU-specific educational in-service (mean difference of -35.8 vs. +7.6, defined daily doses per 1,000 patient-days, p=0.01) and removal of vancomycin as routine surgical prophylaxis for cardiac surgery (mean difference -66.9 vs. +4.2 defined daily doses per 1,000 patient-days, p=0.01). In the multivariable
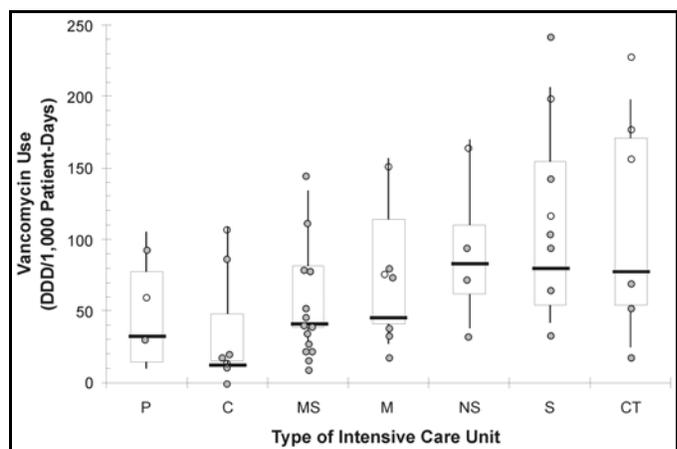
Figure 1. Boxplot of benchmark data of vancomycin use at all Phase 2 Project Intensive Care Antimicrobial Resistance Epidemiology (ICARE) hospitals (n=113 intensive-care units [ICUs]) in October 1997, by type of ICU (18). ICU types include pediatric (P), coronary (C), combined medical-surgical (MS), neurosurgical (NS), surgical (S), and cardiothoracic (CT). For each type of ICU, boxes represent rates of vancomycin use at the 25th–75th percentiles (interquartile range), and ends of vertical lines represent values at the 10th–90th percentiles. Horizontal lines represent median values in each ICU type. Additionally, plotted circles represent the rate of vancomycin use in the pre-intervention period (1996–1997) in the 50 ICUs participating in the intervention study, and open circles represent the 10 ICUs reporting a prescriber practice change identified in the specific unit (i.e., ICU-specific practice change) (1 burn ICU not shown). DDD, defined daily doses.

Table. Prescribing practice changes implemented in response to benchmark data intervention, and mean rate of vancomycin use[a] before and after intervention, 50 Project ICARE ICUs, January 1996–July 1999[b]

| | No. of ICUs (%) | Vancomycin use before and after benchmark data intervention | | | | |
| | | Change absent | | Change present | | |
Vancomycin use prescribing practice change	(n=50)	Before	After	Before	After	p value[c]
Hospitalwide[d]	22 (44%)					
Drug use evaluation	19 (38%)	74.2	80.5	105.3	94.1	0.62
Redistributed HICPAC guidelines on VRE	9 (18%)	79.4	84.6	116.0	90.6	0.34
Prior approval of vancomycin required	3 (6%)	87.2	99.4	84.7	67.2	0.25
Unit-specific[d]	11 (22%)					
ICU-specific education on appropriate vancomycin use	9 (18%)	75.9	96.3	83.3	132.1	0.01
Removed vancomycin from surgical prophylaxis	3 (6%)	82.0	82.2	85.9	149.1	0.01

[a]Defined daily doses per 1,000 patient-days.
[b]Abbreviations: ICARE, Intensive Care Antimicrobial Resistance Epidemiology; ICU, intensive-care units; HICPAC, Healthcare Infection control Practices Advisory Committee; VRE, vancomycin-resistant enterococci.
[c]Paired t-test.
[d]Components of each major categories are not mutually exclusive, so one ICU may be represented in several components of each category.

analysis in which data were adjusted for each ICU and changes in MRSA prevalence, ICUs in which unit-specific practices were identified for improvement used, on average, 49 fewer daily doses of vancomycin per 1,000 patient days than did the other ICUs (parameter estimate -48.5; 95% confidence limits -68.8, -28.22; p=0.0001), compared to pre-intervention levels. The ICUs reported a 35%–37% decrease in median vancomycin use (median 132 daily doses of vancomycin per 1,000 patient days for unit-specific education and 149 for removal of prophylaxis) (Table). Analyses were repeated by using the relative change of each parameter rather than difference in rates, with similar results of statistical significance.

VRE and MRSA

Thirty-five (70%) of the 50 study ICUs tested at least 10 isolates of enterococci for vancomycin susceptibility and were included in the calculations of VRE prevalence during both pre- and postintervention periods. During the pre-intervention period, these ICUs reported a median VRE prevalence of 11.7%. Overall, VRE prevalence increased during the postintervention period compared with the pre-intervention period among all study ICUs (median difference +2.3%; range –41% to +32%), although this difference was not statistically significant. However, when compared by type of practice change, the difference in VRE prevalence was significantly lower in ICUs in which unit-specific practice changes occurred, compared with other ICUs (mean difference -7.5% vs. +5.7%, p<0.001). Although many of the ICUs with decreases in vancomycin use reported increases in percent VRE, all the ICUs noting a unit-specific practice change reported decreases in both percent VRE and vancomycin use (Figure 2). Analysis of these data by using either the relative change in percent VRE or vancomycin use obtained results of similar statistical significance. However, since the relative changes were commonly of extreme values (range 0–400%), these are not reported here.

Because vancomycin use is associated with prevalence of MRSA in ICUs (19), we also evaluated temporal trends in MRSA prevalence. During the pre-intervention period, these ICUs reported a median MRSA prevalence of 33.5%. Overall, prevalence of MRSA increased during the postintervention period compared with the pre-intervention period in all study ICUs (mean difference +5.5%; range –22% to +38%; p=0.02). The increase in MRSA prevalence was similar in ICUs reporting unit-specific practice change compared with other ICUs (mean difference +2.7% vs. +7.1%, p=0.39).

Discussion

In this study involving 50 ICUs from 20 hospitals, we evaluated the effect of inter-institution benchmarking of vancomycin use on reducing vancomycin use and prevalence of VRE. Our study suggests that hospital personnel can use local monitoring data, interpreted in the context of a risk-adjusted external benchmark, to help direct their efforts to reduce excessive use of antimicrobial drugs and reduce antimicrobial resistance. Having access to these data empowered the hospital personnel to make recommendations directed at the specific ICU. Our study further suggests that focused efforts (i.e, ICU specific) may be a more effective means to reduce excessive vancomycin use than hospitalwide activities.

The external benchmarks used were risk adjusted (i.e., stratified by ICU type) to account for the different rates of vancomycin used by different types of ICUs (18). Comparison of local data to a risk-adjusted benchmark should make the comparison more relevant (and more believable) to the ICU staff responsible for prescribing and other patient-care activities. Although several health-care reform proposals suggest some form of interfacility comparisons and public reporting of these types of data (21,22), caution must be exercised by ensuring that the comparisons are risk adjusted. We think part of the success of this study was that risk-adjusted comparisons were
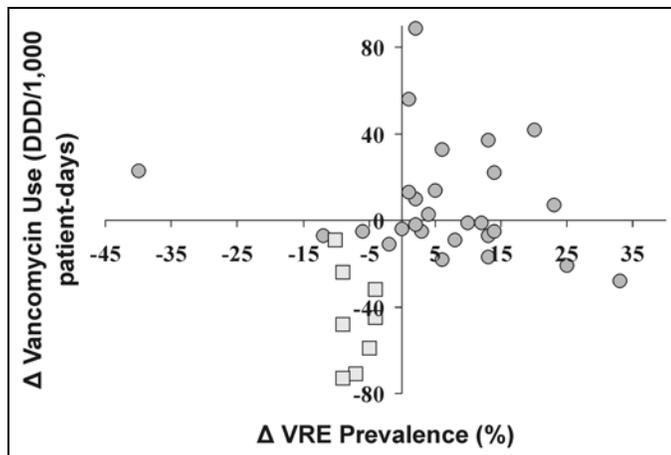
Figure 2. Difference (postintervention period minus pre-intervention) in rate of vancomycin use and prevalence of vancomycin-resistant enterococci (VRE) (%) in 35 intensive-care units (ICUs) testing ≥10 clinical isolates of *Enterococci* spp., Project Intensive Care Antimicrobial Resistance Epidemiology (ICARE), January 1996–July 1999. Squares represent ICUs reporting a prescriber practice change targeted in the specific ICUs (i.e., ICU-specific practice change). DDD, defined daily doses.

provided, rather than an overall single benchmark for all ICU types or all hospitals combined. These comparisons allowed hospital personnel to target unit-specific practice changes to particular ICU areas identified as having an excessive amount of vancomycin used compared with similar types of ICUs in the national benchmark. The reasons unit-specific change in prescriber practice in ICUs were associated with decreases in vancomycin use and VRE are not certain. The impact of the unit-specific practice changes may actually result from engaging local opinion leaders, as has been successfully done in other quality improvement projects (23,24).

Our study suggests that benchmarking rates of antimicrobial use, feedback on these rates, and changes in practice can lead to changes in antimicrobial use. However, the use of some overlapping practice changes and the absence of randomization may limit the ability to generalize the specific practice changes described in this study. In addition, hospitals began activities to reduce vancomycin use through changes in prescriber practice independent of the investigation. In fact, several of the ICUs that used unit-specific changes had the highest rates of vancomycin use, and this excessive use may have made the ICU staff more receptive to the change. These identified changes may not have been successful if implemented in ICUs in which the usage of vancomycin had not been as excessive. This theory may be true but does not detract from the major finding of this study: participation in a monitoring program with comparisons to a valid benchmark provided useful data and allowed the hospital to implement an effective change in practice. In addition, the retrospective nature of ascertaining the description of changes in prescriber practice may involve some recall bias. However, this study demonstrates how a monitoring system provides the tools for hospitals to make rational, valid decisions about initiating activities to change prescribing practices of vancomycin. One aspect of a quality improvement project that was missing from this study was the ability of the infection-control staff to share quality improvement protocols or ideas with other institutions participating in the monitoring system, as has been reported in other quality improvement studies using benchmarking (9).

Our study suggests that interpreting local data in the context of a risk-adjusted benchmark can aid in quality improvement decisions. Many of the study hospitals are continuing to voluntarily report data on antimicrobial use and antimicrobial resistance to CDC's NNIS system as part of a continued quality improvement process. As hospital information systems become more automated, aggregating data such as these should become commonplace. Understanding how to best benchmark and respond to these data will be critical in our efforts to reduce antimicrobial-resistant infections.

Dr. Fridkin is a medical epidemiologist in the Division of Healthcare Quality Promotion (formerly the Hospital Infections Program), National Center for Infectious Diseases, Centers for Disease Control and Prevention. He works primarily on antimicrobial-resistance initiatives, including surveillance of antimicrobial resistance in health care, identification and control of antimicrobial-resistant infections, and outbreak investigations involving new and emerging antimicrobial-resistant pathogens in health-care settings.

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Address for correspondence: Scott K. Fridkin, Division of Healthcare Quality Promotion, MS A35, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30333, USA; fax: 404-639-2647; e-mail: skf0@cdc.gov

Prevalence, Distribution, and Host Range of *Peste des petits ruminants virus*, Turkey

Aykut Özkul,* Yilmaz Akca,* Feray Alkan,* Thomas Barrett,*† Taner Karaoglu,*
Seval Bilge Dagalp,* John Anderson,*† Kadir Yesilbag,* Can Cokcaliskan,*
Ayse Gencay,* and Ibrahim Burgu*

Peste des petits ruminants virus (PPRV, genus *Morbillivirus*), which causes a severe disease in sheep and goats, has only recently been officially declared to be present in Turkey. We carried out a study to determine the prevalence, distribution, and host range of PPRV in Turkey. A total of 1,607 animals, reared in 18 different locations, were monitored for the presence of antibodies to PPRV and the related virus of large ruminants, *Rinderpest virus* (RPV). Only two farms had animals that were free of antibody responses to either disease. Prevalence for PPRV infection varied (range 0.87%–82.6%) and was higher in sheep (29.2%) than in goats (20%). The overall antibody responses to PPRV and RPV were 22.4% and 6.28%, respectively. Two PPRVs of lineage 4, which comprises many other PPRVs whose origins are in the Middle East, the Arabian Peninsula, and southern Asia, were isolated from Turkish sheep.

P*este des petits ruminants virus* (PPRV) is a morbillivirus that primarily infects sheep and goats. The virus is present in Africa (1–3), the Middle East (4), the Arabian Peninsula (5), and southern Asia (6,7) and is closely related to *Rinderpest virus* (RPV), *Canine distemper virus,* and human measles virus (8). Infection with PPRV results in an acute, highly contagious disease characterized by fever, anorexia, necrotic stomatitis, diarrhea, purulent ocular and nasal discharges, and respiratory distress (9,10). Infection rates in sheep and goats rise with age, and the disease, which varies in severity, is rapidly fatal in young animals (10,11). As with other morbillivirus infections, PPRV needs close contact between infected and susceptible animals to spread (10). The two ruminant morbilliviruses, PPRV and RPV, have common antigens demonstrable in a variety of serologic test systems, and they also show a degree of cross-neutralization (12,13). Originally PPRV was considered a variant of RPV adapted to small ruminants; however, the two viruses have separate epizootiologic cycles in nature, and each exists in its own right (14,15).

PPRV infection has only recently been officially reported in Turkey, in September 1999 (16,17), but some reports indicate it was present before then (18,19). The objectives of our research were to determine the seroprevalence of PPRV infection in cattle, sheep, and goats; determine the regional distribution of PPRV in Turkey; isolate and characterize the Turkish virus; and compare its genome sequence with those of other PPRV sequences in the sequence database maintained at the World Reference Laboratory, Pirbright, United Kingdom.

Materials and Methods

Animals Used in the Study

Domestic ruminant species (cattle, sheep, and goats) from throughout Turkey were examined for virus-specific antibodies. The sampling procedure depended on the presence of suspected infection and focused on two groups of animals. The first included 193 sheep that local authorities reported as having clinical signs of PPRV infection. These animals were examined, blood samples were collected, and any animals with signs of disease were sampled by swabbing for virus isolation. Cattle grazing with sheep or goats were also sampled to monitor for antibodies to the two viruses. The second group consisted of 1,414 animals randomly selected for serologic screening for PPRV and RPV antibodies from herds near the flocks of sheep and goats in which PPRV-like infection was reported. The numbers of serum samples collected from sheep, goats, and cattle were 884, 209, and 321, respectively.

Tests for PPRV- and RPV-Specific Antibodies

Competitive enzyme-linked immunosorbent assays were performed as described in the manual of Peste des Petits Ruminants enzyme-linked immunosorbent assay (ELISA) kit (20) and the Office International des Epizooties Manual of Standards (9). Each serum sample, regardless of the species from which it was obtained, was tested for the presence of antibodies to RPV and PPRV.

Virus Isolation Material and Infection of Cell Cultures

A total of 328 field samples, including heparinized blood, organ (lung), and swab specimens, were cultured to obtain virus isolates. The processed samples were spread onto Vero cells seeded in rolling culture tubes. The cells were grown in Dulbecco's modified Eagle medium enriched with 5% fetal

*Ankara University, Ankara, Turkey; and †Institute for Animal Health, Pirbright Laboratory, Surrey, United Kingdom

bovine serum as a regular culture medium. The cell culture media were changed every 2 days and the inoculated cells observed for 12–14 days. The positive culture tubes were frozen at -80°C when the cytopathic effect (CPE) was 90%, and virus stocks were prepared from the positive samples.

Detection of PPRV RNA

Detection of PPRV RNA by reverse transcription-polymerase chain reaction (RT-PCR) was performed as described (21). PCR amplification was carried out by with a PPRV-specific primer set (PPRVF1b: 5´AGTACAAAAGATTGCTGA TCACAGT and PPRVF2d: 5´GGGTCTCGAAGGCTAGGCC CGAATA) selected from the F protein gene sequence, which is expected to amplify a 448-bp DNA product. RT-PCR products were digested by using *Eco*RI at 37°C for 1 hour. Samples were then analyzed on 1.7% agarose gels to determine the cleavage patterns of the amplicons. DNA products obtained with PPR F1b and F2d primers were sequenced by using a T7 polymerase-based commercial kit (Pharmacia Diagnostics AB, Uppsala, Sweden) with ^{35}SdATP as the radiolabel.

Phylogenetic Analysis

Sequence data were analyzed with the GCG (Genetics Computer Group Inc., Madison, WI) package. The nucleic acid sequences obtained from PCR products were aligned with known sequences from representatives of the *Morbillivirus* genus, and the phylogenetic tree was generated with the DNA-DIST and FITCH programs of the PHYLIP 3.73 software (22).

Results

Clinical Findings

Animals with clinical signs of PPRV were detected in 11 provinces (Table 1). In many cases, inspections of flocks confirmed PPRV-suspect cases reported by local veterinarians or identified symptoms indicative of PPRV infection. Most clinical cases were characterized by excessive oculonasal discharge, mild ulcerative stomatitis, dyspnea, and coughing. Severe mucosal eruptions and intestinal signs were not detected.

Serologic Status of Sampled Animals

A total of 1,607 animals from 18 farms were sampled for antibodies to PPRV and RPV (Figure 1). Only two farms (Cihanbeyli and Amasya) had no animals with antibodies specific to either virus. The overall percentages of antibody response to PPRV and RPV were 22.4% and 6.28%, respectively (Table 2). Prevalences of PPRV infection varied between flocks, ranging from 0.87% to 82.60%; however, these figures may not be accurate because of the small sample sizes. In general, the level of PPRV infection was higher in sheep; however, the highest seroprevalence (82.6%) was found in goats in Sakarya Province, where two PPRV isolates were identified from sheep during this project. Of 1,077 sheep examined, 315 (29.2%) were seropositive for PPRV and 1.2%

Table 1. *Peste des petits ruminants virus* (PPRV)–specific antibody prevalence in animals with clinical symptoms indicative of PPRV, Turkey

Location	Animal	Animals with PPRV-suspected symptoms		
		No.	PPRV positive	%
Batman	Sheep	8	7	87.5
Denizli	Goat	10	6	60.0
Cihanbeyli	Sheep	8		
Amasya	Sheep	20		
Sakarya	Sheep	19	3	15.8
Eskisehir	Sheep	5	4	80.0
Malatya	Sheep	3	2	66.6
Sivas	Sheep	23	6	26.0
Isparta	Sheep	32	32	100.0
Aydin	Sheep	42	10	23.8
Van	Sheep	43	40	93.0
Total		213	110	51.6

for RPV. The 10 RPV-seropositive sheep in Bursa Province (in a flock with no clinical PPRV) were reported to have been vaccinated against RPV, while only 1 sheep in Konya was found to have seroconverted, probably following natural infection with RPV before 1999. The overall occurrence of PPRV infection in cattle was 15.57% (a total of 50 animals), and approximately 27% of cattle were antibody positive for RPV, indicating previous exposure to the virus either by natural infection or, most probably, by vaccination, since all cattle in the study were >6 months of age.

The study showed no substantial relationship between the occurrence of PPRV infection and geographic location. Although the main portal of entry of the disease is thought to be in the southeastern part of Anatolia, distribution of the prevalence values did not show a clear pattern across the country, and the disease was detected in varying percentages in almost every region studied (Figure 1).
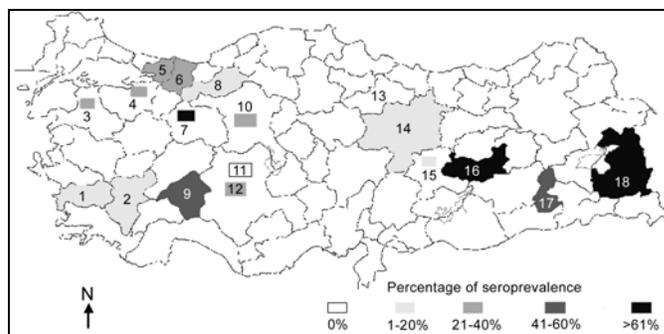
Figure 1. Areas of Turkey sampled to detect the presence of infection with *Peste des petits ruminants virus* and *Rinderpest virus*. Numbers in parentheses indicate the number of serologic test materials collected from each location. Rectangles indicate a single outbreak; shaded provinces had multiple outbreaks. Key: 1, Aydin (100); 2, Denizli (164); 3, Balikesir (40); 4, Bursa (40); 5, Kocaeli (100); 6, Sakarya (100); 7, Eskisehir (5); 8, Bolu (160); 9, Isparta (100); 10, Ankara (20); 11, Cihanbeyli (75); 12, Konya (50); 13, Amasya (20); 14, Sivas (109); 15, Malatya (3); 16, Elazig (272); 17, Batman (50); 16, Van (199).

Table 2. Analysis of antibody response against *Rinderpest virus* (RPV) and *Peste des petits ruminants virus* (PPRV), by species, Turkey

Species	Yr of sampling	No. of sera	No. (%) of animals with antibodies to	
			PPR	RPV
Sheep	1999–2000	1,077	315 (29.20)	13 (1.20)
Goats	1999	209	42 (20.00)	1 (0.47)
Cattle	1999–2000	321	3 (0.90)	87 (27.10)
Total		1,607	360 (22.40)	101 (6.28)

Virus Isolation

A total of 328 samples were spread onto Vero (African Green Monkey Kidney) cells. Two nasal swab samples (Sakarya 1 and Sakarya 2), from sheep in Sakarya Province, showed CPE on Vero cells. The CPE was observed on day 3 after inoculation and was initially characterized by the formation of rounded cells; later, syncytia developed. RT-PCR was performed on cell culture supernatants after the first passage on the Vero cells. The expected amplification product of 448 bp was observed by using RNA prepared from culture supernatants from only the two samples (data not shown). Restriction fragment length polymorphism analysis of the RT-PCR products indicated nucleotide substitutions in the *Eco*RI recognition sequence site in the amplified genome region of the isolates. While the PPR vaccine strain (Nigeria 75/1) produced, as expected, two fragments of 202 bp and 246 bp on cleavage with *Eco*RI, isolates Sakarya 1 and Sakarya 2 were not digested by this restriction enzyme (data not shown). Partial sequencing of the F protein–coding region of the two PPRV isolates showed them to be identical (GenBank accession number AF384687). The Turkey 2000 sequence was then aligned with the sequences of other PPRV isolates from around the world. Figure 2 shows the inferred phylogenetic relationship between the isolates recovered in this research and other PPRVs. The Turkish isolates belonged to PPRV lineage 4 (7), which originates in the Middle East, Arabia, and southern Asia.

Discussion

We investigated the prevalence, host range, and distribution of PPRV in small private farms in Turkey. We also demonstrated the presence of the disease by observing animals in the field and by isolating virus from clinical specimens. This wide-ranging survey is the first to be carried out on this disease in Turkey. PPRV infection has only recently been officially declared to be present in Turkey in the Elazig Province in eastern Anatolia (16,17). Our research provided valuable data on the serologic status of the three domestic ruminant species (cattle, sheep, and goats) with respect to PPRV. Infection with PPRV was demonstrated in 16 of 18 farms we sampled (except for Cihanbeyli and Amasya). On a flock basis, the highest virus prevalence (82.6%) was in goats in Sakarya, where two isolates were identified from sheep. The second highest prevalence (80%) was in sheep in Eskisehir, followed

by 72% in sheep in Van Province and 66.6% in sheep in Malatya Province. Van and Malatya Provinces are in southeastern Turkey near the Iranian border; the remaining provinces are mainly in central Anatolia (Figure 1). Variation in prevalence is probably related to the intensity of trade of illegally imported small ruminants (23).

The prevalence of the disease was as high as 28.5% in sheep and goats reared in small private flocks, and the disease was found in almost every region across Turkey. Occurrence of infection did not vary substantially by geographic locations of the livestock tested. Although the presence of PPRV infection in Turkey has been reported before (18,19), the impact of the disease on production of livestock animals has not previously been investigated. The overall prevalence of PPRV was 22.4% of the ruminant population. These results indicate much lower prevalence than the 88.3% reported by Tatar (19). However, if the overall percentage of PPRV infection takes into account animals reported as having clinical signs, the level increases to 51.6% (Table 1). Another ruminant morbillivirus infection, RPV in cattle, caused great economic losses from the deaths or slaughter of affected (or suspected infected) animals in Turkey in recent years (24). Because PPRV and RPV are antigenically related, the attenuated RPV vaccine has been used to protect small ruminants against PPRV. According to anecdotal reports from the field, veterinarians and animal
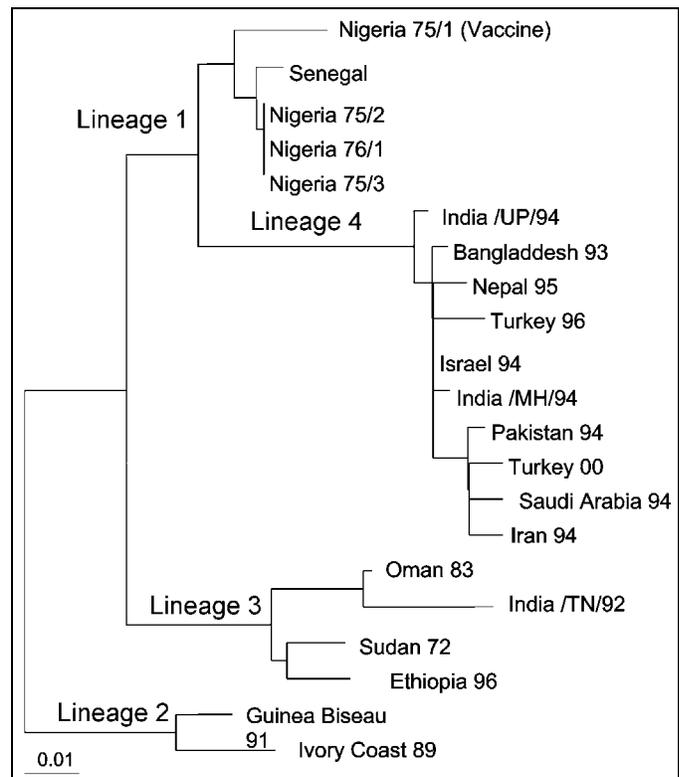
Figure 2. Phylogenetic relationship of the Peste des petits ruminants viruses isolated in Turkey in 2000 to other virus isolates. The tree is based on partial sequence data from the fusion (F) protein gene (7) and was derived by using the PHYLIP DNADIST and FITCH programs (22). Branch lengths are proportional to the genetic distances between viruses and the hypothetical common ancestor at the nodes in the tree. The bar represents nucleotide substitutions per position.

owners widely used the RPV vaccine to protect small ruminants against PPRV infection in some parts of Turkey before RPV vaccination was stopped in the year 2000. This might be one reason for the lower percentage of PPRV-positive animals found in this study.

Cattle act as dead-end hosts for PPRV and show no clinical signs of infection. Nevertheless, they develop a humoral immune response to PPRV that protects them against natural or experimental challenge with virulent RPV (12). In our study, the percentage of natural PPRV infection in cattle was low (0.9%), and all these were in cattle that had contact with infected sheep flocks. Cattle seropositive for PPRV could cause confusion in monitoring for antibodies to RPV after a vaccination campaign to eradicate RPV. Natural infection of cattle with PPRV might prevent the immune response to the RPV vaccine because the PPRV-specific antibodies could neutralize the live attenuated vaccine virus. The cattle would still be protected from subsequent RPV challenge by heterologous PPRV antibody but would register as seronegative when tested in the RPV competitive ELISA. This false value could lead to a low estimation of herd immunity to RPV or suggest that the vaccination coverage was inadequate (12). This risk is high in mixed breeding systems, such as the small-scale production units common in rural regions of Turkey. On the other hand, 14.6% of the cattle had antibodies to both viruses (PPRV and RPV). This finding may indicate that in some cases no interference occurs. Another possible reason is the cross-reactivity of the PPRV assay for antibodies to RPV, as noted by Anderson et al. (25).

Only two PPRVs were isolated—from nasal swab samples of two sheep from Sakarya Province. The reason for the poor success in isolating virus could be the nature of the samples. In previous studies, virus isolations were made from spleen, mesenteric lymph nodes (7,26), or intestinal epithelial smears (15) collected during necropsy of affected animals by inoculation onto Fetal Lamb Kidney (FLK) (19,26) or Vero (15,19) cells. In our study, however, most samples were taken from surviving animals that may have been past the clinical phase of the disease, when the virus is secreted, and so were not likely to yield virus isolates from swabs. Moreover, owners of many sick animals did not grant permission to euthanize them, so internal organs were not available for gross pathologic analysis in most cases. According to a previous study (19), FLK and Vero cells are equally susceptible to PPRV; thus, the use of Vero cells was probably not a factor in our poor success with virus isolation.

Our use of molecular epidemiologic techniques provided data that suggest cross-border transmission into Turkey of PPRV infection that is actively circulating in neighboring countries. The viruses we isolated are PPRV lineage 4, which includes viruses whose origins are in the Middle East, Arabia, and south Asia (7). Because of its geographic location, Turkey has borders with countries where many economically important infectious diseases are endemic. Thus, one of the neighboring countries in the Middle East region is most likely the source of infection. Since the terrain of eastern and southeastern Anatolia permits uncontrolled animal movement, restricting the spread of infectious diseases into the country has been difficult. Therefore, the importance of PPRV as a threat to livestock should be considered, together with other economically important diseases, and measures taken to prevent the import and subsequent spread of such diseases.

Dr. Özkul is a faculty member in the Virology Department of the Veterinary School at Ankara University. His main research interests are the molecular epidemiology of morbilliviruses and the neuropathology of herpes and morbillivirus infections.

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Address for correspondence: Aykut Ozkul, Ankara University, IrfanBastug Cd. Diskapi, 06110 Ankara, Turkey; fax: 90 312 3164472; e-mail: Aykut.Ozkul@veterinary.ankara.edu.tr
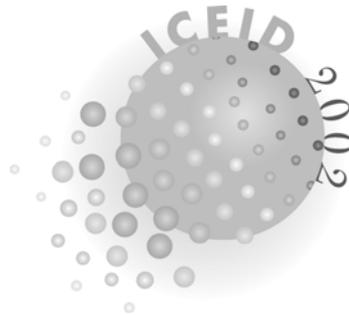
Antibiotic Resistance of Gram-Negative Bacteria in Rivers, United States

Ronald J. Ash,* Brena Mauck,* and Melissa Morgan*

Bacteria with intrinsic resistance to antibiotics are found in nature. Such organisms may acquire additional resistance genes from bacteria introduced into soil or water, and the resident bacteria may be the reservoir or source of widespread resistant organisms found in many environments. We isolated antibiotic-resistant bacteria in freshwater samples from 16 U.S. rivers at 22 sites and measured the prevalence of organisms resistant to β-lactam and non-β–lactam antibiotics. Over 40% of the bacteria resistant to more than one antibiotic had at least one plasmid. Ampicillin resistance genes, as well as other resistance traits, were identified in 70% of the plasmids. The most common resistant organisms belonged to the following genera: *Acinetobacter*, *Alcaligenes*, *Citrobacter*, *Enterobacter*, *Pseudomonas*, and *Serratia*.

The presence of antibiotic-resistant bacteria in freshwater sources throughout the world has been documented (1–5). Selection of resistant organisms in nature may result from natural production of antibiotics by soil organisms, runoff from animal feed or crops, or waste products from treated animals or humans (6–10). Natural reservoirs of resistance genes may provide a source of transferable traits for emerging pathogens (6,11). The prevalence, nature, properties, and origin of such reservoirs in U.S. rivers have not been studied on a national scale. We frequently found organisms resistant to naturally occurring and human-modified antibiotics in U.S. rivers. A large proportion of the resistant organisms were found to contain plasmids with resistance traits.

Methods

Sterile pipettes were used to collect triplicate 10-mL water samples at each site. Samples were collected in city limits at all locations; sites were usually 1 mile downstream of the downtown area. Each sample was collected at a depth of approximately 15 cm. Temperature and pH measurements were also made at the time of sampling. All samples, either undiluted or diluted in Luria-Bertani (LB) broth (12), were plated (0.1 mL, spread plate) on LB agar, some with and some without ampicillin (150 μg/mL) at the collection site. Ampicillin was used to select for potential β-lactamase producers. Water samples were not stored or concentrated before plating. Plates were incubated at 30°C–32°C for 24 hours before the total number of bacteria and ampicillin-resistant bacteria were estimated. Variations in the number of CFU and ampicillin-resistant CFU did not differ (p=0.01) in a group of the triplicate samples plated at a particular time. Ampicillin-resistant isolates were picked to master LB plus ampicillin plates until further tested against additional antibiotics.

National Committee for Clinical Laboratory Standards (NCCLS) procedures and evaluation methods were used (13).

Briefly, isolates were grown in LB plus ampicillin broth until the turbidity of a 0.5 MacFarland standard was reached. Cultures were swabbed on Mueller-Hinton agar, and antibiotic discs (Becton Dickinson and Co., Cockeysville, MD) were added. The antibiotics used were cefotaxime, ceftazidime, amoxicillin plus clavulanic acid, cephalothin, imipenem, kanamycin, streptomycin, chloramphenicol, tetracycline, and ciprofloxacin. MIC were determined with E test strips (AB Biodisk North America, Piscataway, NJ) under NCCLS conditions as described by the manufacturer. Isolates showing complete resistance to at least one antibiotic other than ampicillin were frozen in LB plus 10% dimethyl sulfoxide at -78°C and used as stocks for further testing. Freezing was preferred since isolate storage in the refrigerator for >2 weeks frequently resulted in loss of cultures. Nitrocefin discs (Cefinase, BD Microbiology Systems, Sparks, MD) were placed on bacterial cultures growing on Mueller Hinton agar and observed for the color change indicative of hydrolysis.

Organisms were grown overnight in LB with ampicillin broth with shaking. Two methods were used for every isolate examined: the boiling method (14) and alkaline lysis (15). Agarose gel electrophoresis in 0.7% agarose (GTG, FMC Corp., Rockland, ME) with 1X Tris/acetate/EDTA buffer (12) was used to determine the presence and size of plasmids. Plasmids were purified by removal of unstained gel slices and centrifugal elution through polyester fiberfill plugs (16).

The presence of resistance markers on purified plasmids was determined as follows: electrocompetent *Escherichia coli* (TOP 10 cells, Invitrogen, Inc., Carlsbad, CA) was mixed with 5 μL of pure plasmid DNA and subjected to 1.8 kV in a Bio-Rad Pulser (Bio-Rad Laboratories, Hercules, CA). Electroporated cells (cells with DNA physically introduced) were mixed with SOC medium (12) and incubated for 60 minutes at 37°C before plating on LB with ampicillin plates. Transformants were picked and checked for additional resistance traits as described above.

*Washburn University, Topeka, Kansas, USA

Results

The number and range of ampicillin-resistant bacteria recovered at the 22 sites sampled in the past 3 years are shown in Table 1. Fourteen of the rivers were sampled more than once. Considerable variations in the total number of CFU and ampicillin-resistant organisms were encountered. The MIC for ampicillin was >256 for 98% of the ampicillin-resistant organisms tested. This high level of resistance, found in all rivers tested, was not unexpected since initial plating was on LB plates containing 150 µg/mL of the antibiotic. No apparent correlation existed between numbers of culturable or ampicillin-resistant organisms and temperature (range 0°C–28°C) or pH (range 7.2–8.7) in any of the rivers tested (data not shown). The number of resistant isolates growing on MacConkey agar was >90% for most samples. The ampicillin-resistant isolates were predominantly gram-negative nonlactose-fermenters (data not shown). Oxidase testing and biochemical characterization indicated that the major genera of these bacteria were *Acinetobacter, Alcaligenes, Citrobacter, Enterobacter, Pseudomonas,* and *Serratia. Klebsiella* and *Proteus* were also isolated but less frequently than the other organisms.

The resistance of ampicillin-resistant isolates to other β-lactam antibiotics is presented in Table 2. Organisms resistant to cefotaxime, ceftazidime, and imipenem were detected at a number of sites. This finding prompted us to plate water samples on LB medium with cefotaxime (60 µg/mL). Cefotaxime-resistant bacteria were readily isolated from all rivers tested on this selective medium (Table 3). Of these cefotaxime-resistant organisms, 20% to 30% were gram-positive, spore-forming rods, which appeared to belong to the genus *Bacillus*. Although these gram-positive organisms may be important as reservoirs of resistance genes, only those cefotaxime-resistant isolates growing on MacConkey agar and shown to be gram negative were used for further analysis. Of the gram-negative cefotaxime-resistant organisms, 87% had a cefotaxime MIC >256. The remaining 13% had an MIC >48. Every cefotaxime-resistant isolate was capable of hydrolyzing nitrocefin, indicating the presence of β-lactamase. Many of the cefotaxime-resistant isolates were also resistant to ceftazidime (Table 4). Sixty-one per cent of the ceftazidime-resistant organisms (71 isolates tested) had a ceftazidime MIC >256, while 17% had an MIC of 12 to 192. Most (>80%) of the cefotaxime-resistant and ceftazidime-resistant organisms were identified as *Pseudomonas*.

Table 1. Ampicillin-resistant bacteria, U.S. rivers

River	N[a]	$\log_{10}$ CFU/mL[b]	% ampicillin-resistant
Arkansas-Little Rock, AR	2	3.09–3.37 (3.25)	6.6–21.0
Arkansas-Wichita, KS	4	3.09–4.05 (3.66)	10.4–25.7
Canadian-Oklahoma City, OK	2	3.03–4.36 (4.08)	22.6–24.1
Chattahoochee-Atlanta, GA	2	2.32–2.66 (2.52)	34.5–38.4
Chicago-Chicago, IL	1	4.21	26.9
Colorado-Glenwood Springs, CO	1	2.56	59.2
Cuyahoga-Cleveland, OH	1	3.45	26.4
Des Moines-Des Moines, IA	3	2.66–3.81 (3.40)	13.7–34
Hudson-New York, NY	3	2.94–4.06 (3.80)	10.1–36.6
Kansas-Topeka, KS	24	2.86–4.15 (3.67)	4.9–52.5
Mississippi-New Orleans, LA	1	3.09	5.9
Mississippi-Minneapolis, MN	2	2.89–3.22 (3.09)	19.7–23.7
Mississippi-St. Louis, MO	4	3.93–4.61 (4.31)	6.7–73.0
Missouri-Parkville, MO	7	3.28–4.70 (4.08)	6.1–21.5
Ohio-Cincinnati, OH	6	2.86–4.80 (4.15)	20.0–53.0
Ohio-Louisville, KY	4	2.59–3.70 (3.22)	12.4–20.0
Ohio-Pittsburgh, PA	1	2.53	15.7
Ohio-Wheeling, WV	1	2.76	32.0
Platte-Grand Island, NE	3	3.20–3.56 (3.32)	3.5–33.9
Scioto-Columbus, OH	1	2.80	3.9
Wabash-Terre Haute, IN	3	2.81–3.19 (3.04)	17.0–25.0
White-Indianapolis, IN	1	4.36	22.5

[a]Refers to the number of visits to each site.
[b]Total CFU isolated on Luria-Bertani medium; range and mean (in parentheses) are given for sites sampled more than once.

Table 2. Resistance of ampicillin-resistant isolates to other β-lactam antibiotics, U.S. rivers[a]

River	No. Tested	% resistant				
		CEF	CTX	CAZ	IPM	AMC
Arkansas-Wichita, KS	43	—[a]	5	2	5	—
Canadian-Oklahoma City, OK	50	—	6	0	0	—
Chattahoochee-Atlanta, GA	70	93	13	—	—	—
Chicago-Chicago, IL	81	96	9	0	0	—
Colorado-Glenwood Springs, CO	87	94	25	1	10	—
Cuyahoga-Cleveland, OH	79	91	12	6	0	68
Des Moines-Des Moines, IA	63	8	0	0	0	8
Hudson-New York, NY	107	86	9	0	—	—
Kansas-Topeka, KS	199	25	20	8	4	—
Missouri-Parkville, MO	203	73	21	5	1	—
Ohio-Louisville, KY	85	67	4	2	0	10
Ohio-Cincinnati, OH	91	77	0	0	0	37
Ohio-Pittsburgh, PA	54	78	8	9	—	36
Ohio-Wheeling, WV	50	36	0	0	6	0
Platte-Grand Island, NE	245	96	16	2	—	24
Scioto-Columbus, OH	67	86	2	0	—	33
Mississippi-Minneapolis, MN	72	38	0	0	0	38
Mississippi-St. Louis, MO	51	98	4	0	—	57
Wabash-Terre Haute, IN	81	94	7	0	0	—
White-Indianapolis, IN	83	96	39	—	—	—

[a]Abbreviations used: —,not tested; CEF,cephalothin; CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; AMC, amoxicillin+clavalanic acid.

Table 3. Isolation of cefotaxime-resistant bacteria, U.S. rivers

River	log CFU/mL (%)		
	LB[a]	LB+ampicillin	LB+ cefotaxime
Arkansas-Wichita, KS	3.24	2.65 (25.7)	1.50 (1.8)
Canadian-Oklahoma City, OK	4.36	3.74 (24.1)	2.72 (2.3)
Chicago-Chicago, IL	4.21	3.64 (26.9)	2.37 (1.4)
Des Moines-Des Moines, IA	3.81	2.95 (13.7)	2.09 (1.9)
Hudson-New York, NY	2.94	2.46 (32.8)	1.65 (5.1)
Kansas-Topeka, KS	4.49	4.21 (52.5)	3.23 (5.5)
Mississippi-St. Louis, MO	3.99	3.29 (20.1)	2.51 (3.3)
Missouri-Parkville, MO	4.70	3.96 (18.2)	3.29 (3.9)
Ohio-Louisville, KY	2.70	1.86 (14.4)	0.60 (0.8)
Ohio-Cincinnati, OH	2.89	2.20 (20.0)	0.48 (0.3)
Platte-Grand Island, NE	4.23	3.45 (16.7)	2.74 (3.2)
Wabash-Terre Haute, IN	3.19	2.59 (25.0)	1.75 (3.7)

[a] LB, Luria-Bertani broth.

The resistance of ampicillin-resistant isolates to non-β–lactam antibiotics is shown in Table 5. Many organisms in the rivers had resistance to at least one antibiotic other than ampicillin, and a substantial fraction were able to survive a number of antibiotics. One gram-negative organism resistant to ciprofloxacin was found in the 3,011 ampicillin-resistant isolates tested. Gram-positive ciprofloxacin-resistant bacteria were more numerous and were readily isolated.

Two different plasmid isolation procedures were used to analyze the isolates resistant to ampicillin and one other antibiotic. Of the 374 isolates, 167 (44%) contained at least one plasmid. This number probably represents a minimal estimate since the methods used may not be optimal for all species encountered. Plasmids ranged in size from 2 kb to >23 kb. Purified plasmids were checked for their ability to transform *E. coli*. Of the 54 plasmids tested, 38 (70%) contained the gene for ampicillin resistance. Further, 97% of the plasmids with the ampicillin-resistant gene also carried at least one other resistance trait.

Discussion

We found that culturable antibiotic-resistant bacteria were widespread in nonconcentrated water samples from many U.S. rivers. This finding, in and of itself, is not surprising since the intrinsic resistance of many organisms to antibiotics is well documented (17). Bacteria that are resistant to chemically modified and synthesized antibiotics are also widespread in the environment. The use of selective media resulted in the isolation of gram-negative organisms with high levels of resistance (MIC $\geq$256 µg) to the clinically useful β-lactams ampicillin, cefotaxime, and ceftazidime. Organisms resistant to imipenem were also frequently isolated. Nitrocefin hydrolysis data suggest that β-lactamase production is a major mechanism of resistance to ampicillin in river isolates. The resistance

Table 4. Cefotaxime-resistant isolates, U.S. rivers

River	Ceftazidime-resistant/total tested (%)
Canadian-Oklahoma City, OK	16/50 (32.0)
Chicago-Chicago, IL	14/32 (43.7)
Des Moines-Des Moines, IA	19/28 (67.8)
Kansas-Topeka, KS	15/28 (53.5)
Mississippi-St. Louis, MO	41/43 (95.3)
Missouri-Parkville, MO	28/49 (57.1)
Platte-Grand Island, NE	12/73 (16.4)

of natural isolates to ceftazidime and cefotaxime strongly suggests that these organisms may produce extended-spectrum β-lactamases (ESBL), since resistance to these third-generation cephalosporins is considered the single most important indicator of ESBL (18,19). Alternatively, chromosomal AmpC β-lactamases may be responsible for the resistance of *Pseudomonas* to cefotaxine and ceftazidime (19). Distinguishing

Table 5. Resistance of ampicillin-resistant isolates to non-β–lactam antibiotics[a]

River	No. tested	Ampicillin-resistant isolates	
		Ampicillin + 1 (%)	Ampicillin $\geq$1 (%)
Arkansas-Little Rock, AR	80	3 (3.7)[a]	1 (1.2)
Arkansas-Wichita, KS	155	31 (20)	3 (1.9)
Canadian-Oklahoma City, OK	42	6 (14)	2 (4.7)
Chattahoochee-Atlanta, GA	101	21 (20.8)	2 (2.0)
Chicago-Chicago, IL	100	43 (43)	12 (12)
Colorado-Glenwood Springs, CO	100	32 (32)	24 (24)
Cuyahoga-Cleveland, OH	79	28 (35.4)	10 (12.6)
Des Moines-Des Moines, IA	105	50 (47.6)	8 (7.6)
Hudson-New York, NY	108	20 (18.5)	7 (6.5)
Kansas-Topeka, KS	104	44 (42.3)	2 (1.9)
Mississippi-New Orleans, LA	42	10 (23.8)	4 (9.5)
Mississippi-Minneapolis, MN	115	19 (16.5)	7 (6.0)
Mississippi-St. Louis, MO	161	46 (28.8)	10 (6.2)
Missouri-Parkville, MO	182	30 (16.4)	11 (6.0)
Ohio-Cincinnati, OH	144	16 (11.1)	4 (2.7)
Ohio-Louisville, KY	141	22 (15.6)	7 (4.9)
Ohio-Pittsburgh, PA	54	14 (25.9)	5 (9.2)
Ohio-Wheeling, WV	50	2 (4)	0 (0)
Platte-Grand Island, NE	65	11 (16.9)	3 (4.6)
Scioto-Columbus, OH	59	10 (16.9)	3 (5.0)
Wabash-Terre Haute, IN	109	30 (27.5)	12 (11)
White-Indianapolis, IN	106	17 (16)	5 (4.7)

[a] Ampicillin + 1 = resistance to ampicillin and at least one non-β–lactam. Ampicillin $\geq$1 = resistance to ampicillin and 2 or more non-β–lactams. Non-β–lactam antibiotics tested: ciprofloxacin, tetracycline, chloramphenicol, kanamycin, and streptomycin.

between these possibilities will be important in determining transmissibility of β-lactamase resistance.

Many of the organisms resistant to ampicillin and at least one other antibiotic (40%) harbored plasmids. Although two methods were used to isolate plasmids, some bacteria may have been refractory to these procedures or a large or low copy number plasmids were not observed. Resistance to ampicillin and other antibiotics was plasmid-borne, as indicated by electroporation of cells with purified plasmids.

The results presented here have limitations and must be considered in light of the fact that many aquatic organisms are probably nonculturable (20). The bacteria that cannot be cultivated may be part of the reservoir of resistance genes as well. PCR has been used to identify nonculturable bacteria in stream sediments (21). This technique may be used to identify antibiotic resistance genes in nonculturable organisms as well.

Many of the ampicillin-resistant isolates were also resistant to non-β–lactam antibiotics (Table 5). The frequency with which these organisms were found suggests that characterization of resistance genes and the plasmids on which they reside should provide information about reservoirs for antibiotic resistance in the environment.

Dr. Ash is professor of biology at Washburn University. His research interests include mechanisms of antibiotic resistance.

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Address for correspondence: Ronald J. Ash, Department of Biology, Washburn University, Topeka, KS 66621, USA; fax: 785-231-1089; e-mail: zzash@washburn.edu

Bear Canyon Virus: An Arenavirus Naturally Associated with the California Mouse (*Peromyscus californicus*)

Charles F. Fulhorst,* Stephen G. Bennett,† Mary L. Milazzo,* Hugh L. Murray Jr.,‡ James P. Webb, Jr.,† Maria N.B. Cajimat,* and Robert D. Bradley§

Thirty-four rodents captured in southern California were studied to increase our knowledge of the arenaviruses indigenous to the western United States. An infectious arenavirus was isolated from 5 of 27 California mice but none of the 7 other rodents. Analyses of viral nucleocapsid protein gene sequence data indicated that the isolates from the California mice are strains of a novel Tacaribe serocomplex virus (proposed name "Bear Canyon") that is phylogenetically most closely related to Whitewater Arroyo and Tamiami viruses, the only other Tacaribe serocomplex viruses known to occur in North America. The discovery of Bear Canyon virus is the first unequivocal evidence that the virus family *Arenaviridae* is naturally associated with the rodent genus *Peromyscus* and that a Tacaribe serocomplex virus occurs in California.

T he virus family *Arenaviridae* comprises two serocomplexes (1). The lymphocytic choriomeningitis-Lassa (Old World) complex includes lymphocytic choriomeningitis (LCMV), Lassa (LASV), Mobala, Mopeia, and Ippy viruses. The Tacaribe (New World) complex includes Whitewater Arroyo virus (WWAV), Tamiami (TAMV), Allpahuayo (ALLV), Flexal (FLEV), Paraná (PARV), Pichinde (PICV), Pirital (PIRV), Amapari (AMAV), Guanarito (GTOV), Junin (JUNV), Machupo (MACV), Sabiá (SABV), Tacaribe (TCRV), Oliveros (OLVV), and Latino (LATV) viruses. Heretofore the only arenaviruses known to occur in North America were LCMV, WWAV, and TAMV.

Six arenaviruses are known to cause severe disease in humans (1). LCMV is an agent of acute central nervous system disease and congenital malformations; LASV is the agent of Lassa fever in western Africa; and JUNV, MACV, GTOV, and SABV are etiologic agents of hemorrhagic fever in Argentina, Bolivia, Venezuela, and Brazil, respectively. The occurrence of human disease caused by these viruses ranges from sporadic to hyperendemic.

Specific rodents are the principal hosts of the arenaviruses for which natural host relationships have been well characterized (2). The ubiquitous house mouse (*Mus musculus*) is the principal host of LCMV, woodrats (*Neotoma* species) in the southwestern United States are the principal hosts of WWAV, and the hispid cotton rat (*Sigmodon hispidus*) in southern Florida is the principal host of TAMV (2–8).

Antibody to an arenavirus recently was found in California mice (*Peromyscus californicus*) captured in the Santa Ana

Mountains in Orange County, California (9). The purpose of our study was to determine the identity of the arenavirus associated with such mice in southern California.

Methods

Rodents were captured at three sites near the Riverside-Orange County line: the Bear Canyon Trailhead (Riverside County: 33°36.7′N, 117°25.5′W), "El Cariso #1" (Orange County: 33°39.8′N, 117°25.1′W), and "El Cariso #2" (Orange County: 33°39.8′N, 117°25.7′W). The rodent fauna in the study area included the dusky-footed woodrat (*Neotoma fuscipes*), desert woodrat (*N. lepida*), brush mouse (*P. boylii*), California mouse, cactus mouse (*P. eremicus*), deer mouse (*P. maniculatus*), western harvest mouse (*Reithrodontomys megalotis*), California pocket mouse (*Chaetodipus californicus*), agile kangaroo rat (*Dipodomys agilis*), California ground squirrel (*Spermophilus beecheyi*), and Botta's pocket gopher (*Thomomys bottae*) (S.G. Bennett, unpub. data).

Rodents were captured in Sherman traps (H.B. Sherman Traps, Inc., Tallahassee, FL) or Tomahawk live traps (Tomahawk Live Trap Co., Tomahawk, WI). Fifty traps were set at the Bear Canyon Trailhead on November 12, 1998; 20 traps were set at both El Cariso #1 and El Cariso #2 on June 10, 1998. A blood sample was collected from each rodent, and the blood samples and rodent carcasses were shipped on dry ice to the University of Texas Medical Branch. Subsequently, the carcasses and samples of lung, heart, liver, and skeletal muscle were deposited in the Museum of Texas Tech University, and mammalogists at Texas Tech University identified each rodent to species level on the basis of morphologic features of the animal's skin and skull.

The blood samples were tested for antibody to WWAV by using an enzyme-linked immunosorbent assay (9). The test

*University of Texas Medical Branch, Galveston, Texas, USA; †Orange County Vector Control District, Garden Grove, California, USA; ‡County of Riverside, Department of Environmental Health, Hemet, California, USA; and §Texas Tech University, Lubbock, Texas, USA

antigen was a detergent extract of Vero E6 cell monolayers infected with the WWAV prototype strain AV 9310135. The control (comparison) antigen was prepared from uninfected Vero E6 cell monolayers in a manner quantitatively identical to that used to prepare the test antigen. Serial fourfold dilutions (from 1:80 through 1:5,120) of each blood sample were tested against both antigens. The adjusted optical density (AOD) of a blood-antigen reaction was the OD of the well coated with the test antigen less the OD of the corresponding well coated with the control (comparison) antigen. A sample was considered to be antibody-positive if the AOD at 1:80 and 1:320 both were $\geq$0.200 and the sum of the AODs for the series of fourfold dilutions (from 1:80 through 1:5,120) was $\geq$0.750. The criteria for antibody positivity were based on the results of a laboratory study of white-throated woodrats (*N. albigula*) experimentally infected with the WWAV prototype strain AV 9310135 (10). The antibody titer of a positive sample was the reciprocal of the highest dilution of that sample for which the AOD was $\geq$0.200.

A 10% wt/vol crude homogenate of brain tissue from each animal was tested for infectious arenavirus by cultivation in a monolayer of Vero E6 cells (4). Cells scraped from the monolayer on day 13 after inoculation were tested for arenaviral antigen by an indirect fluorescent antibody test. The primary antibody in that test was a hyperimmune mouse ascitic fluid prepared against the WWAV prototype strain AV 9310135.

The nucleotide sequence of a 489-nt fragment of the nucleocapsid protein (NP) gene of isolate A0070039 and the sequence of the homologous fragment of the NP gene of each of the four other viral isolates from rodents included in this study were determined (Table). Subsequently, the nucleotide sequence of a 616-nt fragment of the NP gene of isolate A0070039 was determined. The 616-nt fragment included the entire 489-nt NP gene fragment and represented the genomic region that has been the basis of several comprehensive studies on the phylogeny of the arenaviruses (11–15). Total RNA was

extracted from infected Vero E6 cells by using TRIzol Reagent (Life Technologies, Inc., Rockville, MD). Reverse transcription (RT) and polymerase chain reaction (PCR) amplification of the 489-nt gene fragment were performed with the Access RT-PCR Kit (Promega Corp., Madison, WI) in conjunction with oligonucleotides AVNP3 (5´-TCTTGATGACTATTC-CCTTATGC-3´) and AVNP4 (5´-AACACTGTGGT-TGAGTTTGATAG-3´). RT-PCR amplification of the 616-nt gene fragment was done by using SuperScript II Rnase H⁻ Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA) and PCR SuperMix (Invitrogen Life Technologies) in conjunction with oligonucleotides ARE3´-END, and AVNP7 (5´-TCTGGAGAAGGATGGCC-3´) and AVNP8 (5´-ACATGATACAATCCATCAATGCACAGTG-3´), respectively (16). PCR products of the expected size (535 bp or 661 bp) were purified from agarose gel slices with the QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, CA). Both strands of each purified PCR product were sequenced directly by using the dye termination cycle sequencing technique (Applied Biosystems, Inc., Foster City, CA) in conjunction with oligonucleotides AVNP3 and AVNP4 or AVNP7 and AVNP8. The nucleotide sequences generated in this study were deposited in the GenBank nucleotide sequence database under accession nos. AY093616 and AF497572 through AF497575.

The sequence of the 616-nt fragment of the NP gene of isolate A0070039 was compared with the sequence of the homologous region of the NP genes of WWAV strains AV 9310135, AV 98490013, AV 96010025, AV 96010151, AV 96010024, and AV A0400174, TAMV, the 13 South American arenaviruses, LCMV, and LASV. The GenBank database sequences included in the analyses were accession nos. U52180, AY012711, AY012720, AY012717, AY012710, AY012713, U43690, Y012687, U43687, U43689, K02734, U62561, U43685, U43686, U70802, X62616, U41071, M20304, U43688, U34248, M20869, and AF182272. The predicted

Table. Results of tests for arenaviral infection in 10 antibody-positive or virus-positive rodents, California

Rodent	Species	Date captured	Trap site[a]	Antibody titer[b]	Viral strain
TK 90425	*Neotoma fuscipes*	11/13/98	BCN	$\geq$5,120	—
TK 90430	*Peromyscus californicus*	11/13/98	BCN	$\geq$5,120	—
TK 90435	*P. californicus*	11/13/98	BCN	$\geq$5,120	—
TK 90438	*P. californicus*	11/13/98	BCN	1,280	AV A0070039
TK 90444	*P. californicus*	11/13/98	BCN	$\geq$5,120	—
TK 90779	*P. californicus*	06/11/98	EC1	$\geq$5,120	AV A0060207
TK 90586	*P. californicus*	06/11/98	EC2	$\geq$5,120	AV A0060211
TK 90599	*P. californicus*	06/11/98	EC2	$\geq$5,120	AV A0060209
TK 90785	*P. californicus*	06/11/98	EC2	$\geq$5,120	AV A0060210
TK 90496	*P. californicus*	06/11/98	EC2	320	—

[a]BCN, Bear Canyon Trailhead; EC1, El Cariso #1; EC2, El Cariso #2.
[b]The antibody titer was the reciprocal of the highest dilution of the blood sample for which the AOD was $\geq$0.200 (see text).
—, No viral isolate.

amino acid sequences were aligned by using the computer program CLUSTAL W1.7 (17). The multiple nucleotide sequence alignment was constructed manually based on the results of the amino acid sequence alignment. The analyses of the multiple nucleotide sequence alignment were done by using programs in the computer software package PAUP*, version 4.0b8a (18). The maximum likelihood (ML) analysis used the GTR+I+G model of substitution and the heuristic search option with a gamma shape parameter and transition-transversion ratio (Ti/Tv) of 0.508 and 1.96, respectively. The Modeltest program (19) indicated that the GTR+I+G model of substitution best fit the data for the likelihood analysis. Genetic distances were calculated by using the p model and Kimura two-parameter distance model (20). Sequence identities were calculated by subtracting the uncorrected p model distances from 1.0. The neighbor-joining (NJ) analysis used the Kimura genetic distances. The maximum parsimony (MP) analyses (all characters weighted equally, first and second nucleotide positions only, and amino acid sequences predicted from nucleotide sequences) were restricted to informative characters, with nodal support estimated by bootstrap analysis (21). Bootstrap support for the results of the NJ and MP analyses was based on 1,000 pseudoreplicate data sets generated from the original multiple nucleotide sequence alignment.

Results

Thirty-four rodents (2 dusky-footed woodrats, 5 brush mice, and 27 California mice) were captured and tested for antibody or infectious arenavirus. Antibody to an arenavirus was detected in 1 of 2 dusky-footed woodrats and 4 of 8 California mice captured at the Bear Canyon Trailhead, 1 of 11 California mice captured at El Cariso #1, and 4 of 8 California mice and none of 5 brush mice captured at El Cariso #2. Infectious arenavirus was isolated from 1 California mouse each captured at the Bear Canyon Trailhead and El Cariso #1, 3 California mice captured at El Cariso #2, and none of the 29 other rodents. The five virus-positive animals also were antibody-positive (Table).

Sequence identities among the 489-nt NP gene fragments generated from the five isolates from the California mice ranged from 96.3% to 100.0%, indicating that the isolates are strains of a single virus. When compared with other arenaviruses, the 616-nt NP gene fragment of isolate A0070039 exhibited the highest nucleotide sequence identity and predicted amino acid sequence identity with (in decreasing order) the NP gene of WWAV prototype strain AV 9310135 (72.7% and 82.9%, respectively), TAMV (72.6% and 76.6%, respectively), the 13 South American arenaviruses (ALLV, AMAV, FLEV, GTOV, JUNV, LATV, MACV, OLVV, PARV, PICV, PIRV, SABV, and TCRV: 55.5%-63.5% and 47.8%-62.0%, respectively), and LCMV and LASV viruses (51.5%-51.9% and 42.7%-43.4%, respectively). In the same analysis, nucleotide and amino acid sequence identities between WWAV and TAMV were 73.1% and 80.0%, respectively, and nucleotide and amino acid sequence identities among the 13 South Amer-

ican arenaviruses ranged from 54.9% to 76.5% and 47.3% to 86.2%, respectively. Collectively, these results indicate that the arenavirus isolated from the California mice is distinct from all other Tacaribe complex viruses. The name "Bear Canyon" is proposed to denote the geographic origin of the first isolate of this novel virus, and strain A0070039 is designated as the prototype strain of BCNV.

The results of the ML analysis of nucleotide sequence data (Figure), NJ analyses of Kimura two-parameter genetic distances, and MP analyses of nucleotide and amino acid sequence data all indicated that BCNV, WWAV, and TAMV are monophyletic and phylogenetically distinct from the South American arenaviruses, LCMV, and LASV. Bootstrap support for monophyly of BCNV, WWAV, and TAMV was 100% in both the NJ and MP analyses.
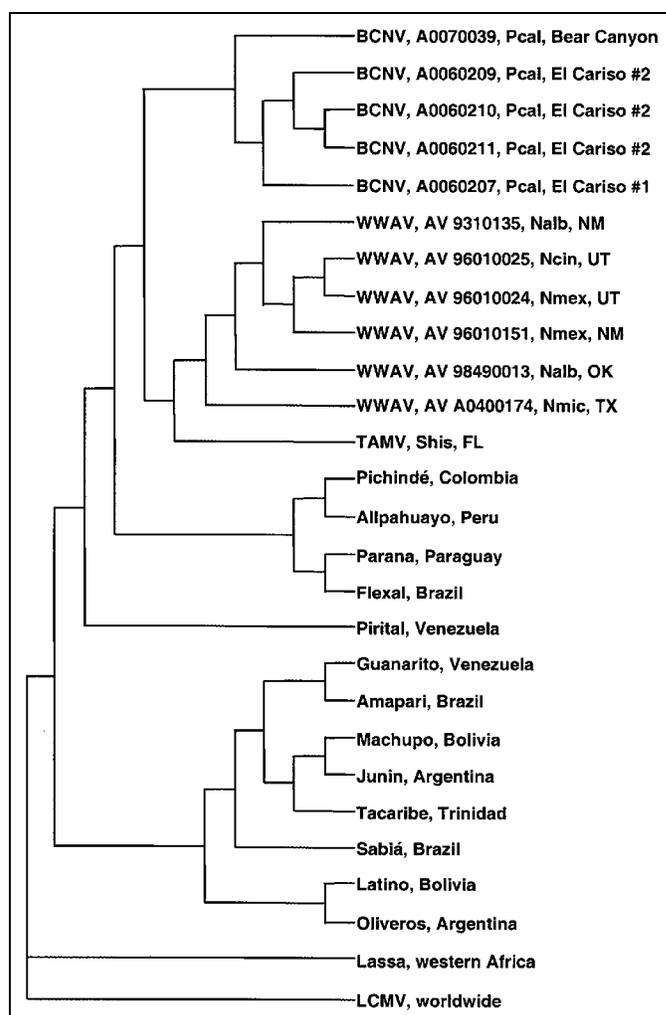
Figure. Phylogenetic relationships among Bear Canyon virus (BCNV) prototype strain A0070039, 4 other BCNV isolates from California mice (*Peromyscus californicus*), Whitewater Arroyo virus (WWAV), Tamiami virus (TAMV), and 15 other arenaviruses, based on a maximum likelihood analysis of a fragment of the nucleocapsid protein gene. The string of characters following "BCNV" or "WWAV" denotes the virus strain. LCMV, lymphocytic choriomeningitis; Pcal, *Peromyscus californicus*; Nalb, *Neotoma albigula*; Ncin, *N. cinerea*; Nmex, *N. mexicana*; Nmic, *N. micropus*; Shis, *Sigmodon hispidus*; NM, New Mexico; UT, Utah; OK, Oklahoma; TX, Texas; FL, Florida.

The ML analysis of nucleotide sequence data and MP analysis of amino acid sequence data placed WWAV in a sister relationship to TAMV and the WWAV–TAMV lineage in a sister relationship to the BCN lineage. However, bootstrap support for the WWAV–TAMV lineage in the MP analysis was low, i.e., 51%. The MP analysis of all nucleotide positions placed BCNV in a sister relationship to WWAV and the BCNV–WWAV lineage in a sister relationship to the TAMV lineage. However, bootstrap support for the BCN–WWAV lineage in that analysis was only 53%. The NJ analyses of nucleotide sequence data (with and without third position bases) indicated that BCNV, WWAV, and TAMV represent a trichotomy in the evolution of the Tacaribe complex viruses. Similarly, the MP analysis of nucleotide sequence data with third position bases excluded indicated that BCNV, WWAV, and TAMV represent a trichotomy in the evolution of the Tacaribe complex viruses. Sequence data representative of a larger fragment of the NP gene or a different region of the arenavirus genome may enable resolution of the phylogenetic relationships among the three North American viruses. Alternatively, the BCNV–WWAV–TAMV trichotomy may be the result of an essentially simultaneous divergence from the last common ancestor of the three North American arenaviruses. In that scenario, it would be difficult, if not impossible, to resolve the relationships among viruses in the North American (BCNV–WWAV–TAMV) lineage.

Discussion

Previous studies of wild rodents in coastal southern California (Los Angeles, Orange, San Bernardino, San Diego, and Ventura Counties) showed antibody to an arenavirus in dusky-footed and desert woodrats, a brush mouse, California mice, a cactus mouse, deer mice, and western harvest mice (3,9). The isolation of BCNV from California mice is the first unequivocal evidence that a Tacaribe complex virus occurs in California and that the virus family *Arenaviridae* is naturally associated with the rodent genus *Peromyscus*. Further work is needed to determine the identity of the Tacaribe complex virus(-es) associated with woodrats, brush mice, cactus mice, deer mice, and western harvest mice in southern California.

In a recent study (5), WWAV was isolated from white-throated woodrats captured in northwestern New Mexico and western Oklahoma, a bushy-tailed woodrat (*N. cinerea*) in southern Utah, Mexican woodrats (*N. mexicana*) in central New Mexico and southern Utah, and southern plains woodrats (*N. micropus*) in southern Texas. The broad geographic association of WWAV with the rodent genus *Neotoma* suggests that WWAV is the arenavirus associated with dusky-footed woodrats in the Santa Ana Mountains.

The high prevalence of infection (50%) in California mice at El Cariso #2 and isolation of BCNV from California mice captured at the Bear Canyon Trailhead and El Cariso #1 indicate that the California mouse is the principal host of BCNV. However, the infections in the California mice could be the result of horizontal virus transmission from dusky-footed woodrats or another rodent that was not well represented in our study.

The geographic range of *P. californicus* extends from central California to San Quintin in Baja California (22). Throughout that range, the California mouse oftentimes is closely associated with middens of dusky-footed woodrats (22). Thus, the antibody-positive woodrat captured at the Bear Canyon Trailhead may have been infected with BCNV as a result of contact with infected California mice. Conversely, the California mice may have been infected with the virus as a result of contact with infected dusky-footed woodrats.

Human disease caused by Tacaribe complex viruses has been studied almost exclusively in South America. The results of our study indicate that there are substantial genetic differences among BCNV, WWAV, and TAMV. The genetic sequence differences and similarities among these viruses should be considered in the development of molecular-based assays for diagnosis of human disease caused by North American arenaviruses.

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Address for correspondence: Charles F. Fulhorst, University of Texas Medical Branch, Department of Pathology, Route 0609, Galveston, Texas 77555-0609, USA; fax: 409-747-2415; e-mail: cfulhors@utmb.edu

Entomologic and Serologic Evidence of Zoonotic Transmission of *Babesia microti*, Eastern Switzerland

Ivo M. Foppa,* Peter J. Krause,† Andrew Spielman,* Heidi Goethert,* Lise Gern,‡ Brigit Brand,§ and Sam R. Telford III*

We evaluated human risk for infection with *Babesia microti* at a site in eastern Switzerland where several *B. microti*–infected nymphal *Ixodes ricinus* ticks had been found. DNA from pooled nymphal ticks amplified by polymerase chain reaction was highly homologous to published *B. microti* sequences. More ticks carried babesial infection in the lower portion of the rectangular 0.7-ha grid than in the upper (11% vs. 0.8%). In addition, we measured seroprevalence of immunoglobulin (Ig) G antibodies against *B. microti* antigen in nearby residents. Serum from 1.5% of the 396 human residents of the region reacted to *B. microti* antigen (≥1:64), as determined by indirect immunofluorescence assay (IgG). These observations constitute the first report demonstrating *B. microti* in a human-biting vector, associated with evidence of human exposure to this agent in a European site.

A malaria-like syndrome due to *Babesia microti* infection has been recognized in parts of the northeastern United States for more than three decades (1,2). This protozoon pathogen was first isolated more than half a century earlier from a Portuguese vole (3); the pathogen has since been detected in small mammals and ticks throughout Eurasia (3,4).

Despite its broad geographic distribution, *B. microti* has not been implicated as a cause of human illness in Europe. A host-specific, rodent-feeding tick, *Ixodes trianguliceps*, is widely regarded as the main enzootic vector on that continent. *I. ricinus*, the most common human-biting tick of Europe, transmits the Lyme borreliosis spirochete, tick-borne encephalitis virus, the agent of human granulocytic ehrlichiosis, and *B. divergens*, but *I. ricinus* was believed to be infected only occasionally with *B. microti* (5). This vector-pathogen association may account for the absence of human disease due to *B. microti* (3,6). However, subadult *I. ricinus* ticks feed abundantly on the reservoirs of *B. microti*, such as voles and mice, and appear to be competent vectors for *B. microti* (7). In fact, recent studies indicate that Swiss residents may have concurrent infection with the Lyme disease spirochete and *B. microti* (8) and that the human population of certain parts of Germany is exposed to *B. microti* (9).

Human exposure to *B. microti* may occur more often in Europe than has been recognized. Accordingly, we assessed the potential of zoonotic transmission in eastern Switzerland, where other *I. ricinus*–transmitted infections are present. In particular, we determined how frequently *B. microti* parasites

infect *I. ricinus* ticks locally, how infection in ticks is spatially distributed, and how frequently the sera of nearby residents react to *B. microti* antigen.

Methods

Tick Collection and *B. microti* Detection in Ticks

To assess local prevalence of *B. microti* in host-seeking nymphal *I. ricinus* ticks, we developed a tick-sampling procedure with high spatial resolution (Figure 1). The roughly rectangular, 0.7-ha field site, Ruetiwis (9° 38′ E, 46° 59′ N), is located on a steep southwesterly slope near Seewis in the lower Praettigau Valley of eastern Switzerland at an approximate mean altitude of 850 m above sea level. The site is characterized by abandoned pastures that are partly overgrown by young stands of deciduous and coniferous trees and bushes, as well as by mature mixed forest. All ticks were collected by flagging in July 1997.
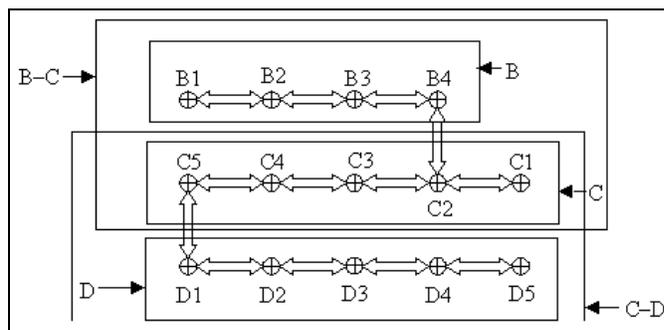
Figure 1. Schematic drawing of the sampling scheme. Distance between sampling points is 30 m. The letters and solid arrows denote the sections used for prevalence estimation. Sampling lines that connect points not belonging to a section are not included in that section (e.g., line B4–C2 is not part of section B).

*Harvard School of Public Health, Boston, Massachusetts, USA; †Connecticut Children's Medical Center and University of Connecticut School of Medicine, Hartford, Connecticut, USA; ‡Université de Neuchâtel, Institut de Zoologie, Neuchâtel, Switzerland; and §Kantonsspital, Blutspendezentrum, Chur, Switzerland

To ensure the quality of tick-derived DNA, pools of 2–11 nymphal ticks were transferred to 0.5-mL microcentrifuge tubes containing 50 μL guanidium thiocyanate solution and stored at room temperature until further processing. Before homogenization with a glass pestle, the solution containing the ticks was incubated at 60°C for 2 h. DNA was extracted from the tick homogenate by the phenol-chloroform method. The resulting DNA pellet was suspended in 30 μL DNAse-free H_2O.

To determine whether *B. microti*–specific DNA was present, the extracted DNA was subjected to polymerase chain reaction (PCR) with the primer pair Bab-1 (5′-ttagtataagcttt-tatacagc-3′) and Bab-4 (5′-ataggtcagaaacttgaatgataca-3′) (10), which targets a 250-bp fragment of the 18s rRNA gene of *B. microti*. After denaturation for 2 min at 94°C, 40 cycles were performed, with 45 sec at 94°C, 45 sec at 55°C, and 45 sec at 72°C, followed by a 7-min final extension. Amplification products were separated on 2% agarose gel in Tris-borate-EDTA buffer, stained with ethidium bromide, and visualized under UV light. To differentiate the sequence of interest from a frequently observed, slightly smaller fragment, purified PCR products were digested with XhoI (Life Technologies, Invitrogen Corp., Gaithersburg, MD). The resulting two fragments specific for *B. microti* migrate in one band on the 2% agarose gel. The corresponding sequence of *B. divergens* lacks the restriction site.

To verify the taxonomic status of the *Babesia* spp. detected in these ticks, we conducted a phylogenetic analysis on a representative sample from which DNA had been amplified (Bab-1/Bab-4). For this purpose we used the primer pair PIRO A and PIRO B, which also targets 18s rDNA but is less specific for *B. microti*; the resulting sequence is longer (400 bp) than the Bab-1/Bab-4 amplicon (11). After PCR amplification, the respective band was excised from the agarose gel, purified with spin columns (Qiagen Inc., Valencia, CA), and sent to the University of Maine sequencing facility for sequence analysis. The resulting sequence was aligned against other *Babesia* spp. listed in GenBank by using Clustal X and consecutive adjustment visually. Phylogenetic analysis was performed by both maximum parsimony (Swofford D. Phylogenetic analysis using parsimony, PAUP*4b61; Sinauer Associates, Inc., Sunderland, MA) and neighbor-joining analyses (12) with *Toxoplasma gondii* (GenBank accession no. X68523) as outgroup. Robustness of the nodes was assessed by bootstrap analysis with 500 bootstrap replicates.

Data Analysis

To accurately estimate the local prevalence of *B. microti* infection in *I. ricinus* ticks, we developed a maximum likelihood method for point estimation as well as a method for calculating confidence intervals. Briefly, the maximum likelihood estimate (MLE) of the prevalence p is based on the likelihood

$$L(\beta) = \left(1 - \frac{Exp(\beta)}{1 + Exp(\beta)}\right)^{N^-} \prod_J \left(1 - \left(1 - \frac{Exp(\beta)}{1 + Exp(\beta)}\right)^j\right)^{n_j},$$

where $\beta = Log\left(\dfrac{p}{1-p}\right)$, $N-$ is the number of ticks in negative pools, J is the set of pool sizes with at least one positive pool, and n_j is the number of positive pools of size j. Test-inversion bootstrap confidence intervals (13) were calculated for the prevalence estimate.

To determine whether infected ticks were clustered in the study site, we tested the null hypothesis $\beta_1=\beta_2=0$ by the model

$$p_k = \frac{Exp(\beta_0 + \beta_1 x_{1k} + \beta_2 x_{2k})}{1 + Exp(\beta_0 + \beta_1 x_{1k} + \beta_2 x_{2k})}, \text{ where } x_{1k} = 1$$

if the $x_{1k} = 0$ pool is from section C and otherwise, and $x_{2k} = 1$ if the pool is from section D and $x_{2k} = 0$ otherwise. If the large sample confidence interval around the MLEs of β_1, β_2 or both does not include 0, evidence for clustering is positive.

Serologic Survey

To determine whether humans may be exposed to *B. microti* in the study area, we recruited 400 blood donors living within 10 km of the field site for a serologic survey of tick-borne zoonoses. Volunteers were recruited for this cross-sectional seroprevalence study during blood drives from December 1997 to May 1998 in towns within a 10-km radius of the study site. This protocol was approved by the Human Subjects Committee of the Harvard School of Public Health (protocol number 9712THEE). The sera of participants who gave their written informed consent were tested by indirect immunofluorescence assay (IFA) as described (14). Antigen slides were prepared from erythrocytes of *B. microti*–infected hamsters (the GI strain, originally derived from a Nantucket Island patient). Sera were first screened by IFA at 1:64 dilution. A panel of sera included all samples reactive in the screening test for which enough serum was available, including samples with borderline reactivity and representative controls. This panel was coded and blindly retested for *B. microti* IFA at the University of Connecticut laboratories, which specialize in *Babesia* serology. An IFA titer ≥1:64 was considered reactive. All reactive sera were titrated to endpoint.

Results

Tick Survey

To verify the identity of the amplified DNA, a phylogenetic analysis was performed. The sequence amplified from our *I. ricinus* ticks, which was deposited in GenBank (accession no. AF494286), differed by l bp from the North American *B. microti* sequence (GenBank accession no. AF231348) and was identical with that of *B. microti* from Slovenia (GenBank accession no. AF373332). Accordingly, our sequence clearly clustered with the European and the American strain of *B. microti* (Figure 2), with concordant results from both maximum parsimony and neighbor-joining analyses. Therefore, the piroplasms detected in *I. ricinus* ticks from Switzerland must be considered *B. microti*.
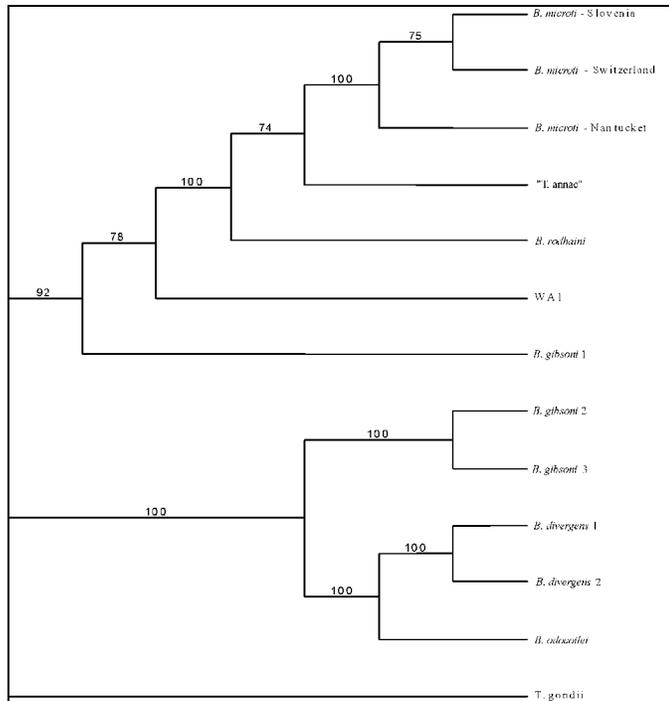
Figure 2. Maximum parsimony bootstrap consensus tree of 18S rDNA. GenBank accession nos.: *Babesia microti*-Slovenia AF373332; *B. microti*-Switzerland AF494286; *B. microti*- Nantucket AF231348; "Toxoplasma annae" AF188001; *B. rodhaini* AB049999; WA1 AF158700; *B. gibsoni* 1 AF158702; *B. divergens* 1 U07885; *B. divergens* 2 U16370; *B. odocoilei* U16369; *B. gibsoni* 2 AF175300; *B. gibsoni* 3 AF175301; and *Toxoplasma gondii* X68523.

We then determined the prevalence of *B. microti* infection in nymphal *I. ricinus* ticks from the study site, on the basis of PCR amplification of *B. microti* in DNA tick pools. Overall, we analyzed 408 ticks in 64 pools. We detected *B. microti*–specific DNA in 14 pools (Table 1). Thus, *B. microti* infection appears to be common in human-biting ticks at this central European study site.

To determine whether the prevalence of *B. microti* is distributed homogeneously within the study site, prevalence of infection in ticks was estimated for selected segments of the sampling grid. The overall prevalence of *B. microti* infection in ticks, as estimated by MLE, was close to 4%. More detailed spatial analysis, however, indicates that the distribution of babesial infection at the site is heterogeneous (Table 2). In the lower portion of the site (section D), >10% of all ticks were infected, while in the upper portion (B–C), prevalence was <1%. Prevalence of infection in ticks was similar in sections B and C (p=0.161), but greater in section D than in sections B and C (p=0.003). Thus, *B. microti* transmission is focal at this site.

Serologic Survey

To determine how frequently humans are exposed to *B. microti* infection in the region around the study site, sera of local residents were screened by IFA for the presence of antibodies to *B. microti* antigen. Most sera (80%) were collected from December through March, when *I. ricinus* is not active.

Table 1. *Babesia microti* infection in nymphal *Ixodes ricinus* ticks as determined by polymerase chain reaction, eastern Switzerland

Sampling point or line	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	No. of pools	No. of ticks
B1	11	11	12	—	—	3	34
B1–2	10	10	10	—	—	3	30
B2	4	3	—	—	—	2	7
B2–3	3	3	3	—	—	3	9
B3	3	—	—	—	—	1	3
B3–4	**8**[a]	8	—	—	—	2	16
B4	1	—	—	—	—	1	1
B4–C2	5	5	4	5	—	4	19
C1	1	—	—	—	—	1	1
C1–2	9	9	9	9	—	4	36
C2	**7**	7	5	—	—	3	19
C2–3	5	5	5	5	5	5	25
C3	10	—	—	—	—	1	10
C3–4	4	—	—	—	—	1	4
C4	4	—	—	—	—	1	4
C4–5	7	6	—	—	—	2	13
C5	8	7	6	—	—	3	21
C5–D1	**8**	7	7	8	—	4	30
D1	**5**	**5**	6	—	—	3	16
D1–2	8	7	—	—	—	2	15
D2	6	6	—	—	—	2	12
D2–3	**5**	**5**	**5**	**6**	6	5	27
D3	4	—	—	—	—	1	4
D3–4	**6**	6	5	—	—	3	17
D4	**11**	**11**	10	—	—	3	32
D4–5	3	—	—	—	—	1	3
D5	0	—	—	—	—	0	0
Total						64	408

[a]Pools in bold are positive; dashes represent the absence of further pools.

Five (1.5%) of 396 human sera were immunoglobulin (Ig) G-reactive against *B. microti* antigen at a titer ≥1:64. All the samples positive on initial testing and one with a borderline result were confirmed on retesting to be reactive at a titer >1:64, while none of the samples with a negative screening result reacted (Table 3). *B. microti*–specific IgM seroreactivity was not found in any of the sera, a finding compatible with the time of the year when most of the sera had been collected. These results indicate that residents of our central European study site are exposed to bites of ticks infected with *B. microti*.

Discussion

B. microti–infected nymphal *I. ricinus* ticks are present at this Swiss study site, and human residents of the area appear to

Table 2. Global and local prevalence estimates of *Babesia microti* infection in nymphal *Ixodes ricinus* ticks as determined by polymerase chain reaction, eastern Switzerland

	Point estimate (%)	95% confidence interval
MLE[a], overall	3.6	0.2 to 9.06
MLE, section B	1.04	0.10 to 9.01
MLE, section C	0.77	0.08 to 8.47
MLE, section D	10.99	6.17 to 17.71
MLE, sections B,C	0.82	0.61 to 2.65
MLE, sections C,D	5.25	2.96 to 10.43

[a]MLE (maximum likelihood estimate) of the point estimates are shown with 95% bootstrap confidence limits.

Table 3. Reactivity of sera tested against *Babesia microti* as determined by indirect immunofluorescent assay

Titer	No. of sera (%)
<1:64	391 (98.7)
1:64	1 (0.3)
1:128	1 (0.3)
1:256	1 (0.3)
1:512	2 (0.5)
Total	396 (100.0)

be exposed to this agent. However, the presence of *B. microti* in *I. ricinus* ticks has been reported only rarely (5).

A phylogenetic analysis clearly demonstrated that the piroplasms found in our study site belong to *B. microti*, rather than to other *Babesia* or *Theileria* species (Figure 2). The detection of *B. microti* DNA in host-seeking nymphal *I. ricinus* ticks may, however, simply reflect "spill-over" from enzootic transmission by the accepted maintenance vector for *B. microti* in Eurasia, the tick *I. trianguliceps* (15). This tick, which does not bite humans, infests small rodents that also are abundantly parasitized by *I. ricinus* (3,16). The prevalence of infection in ticks may be underestimated as only one genome copy is present per parasite in unfed ticks before sporogony. Therefore, low parasite loads may escape detection, thus increasing the specificity of the assay.

In our study, maximizing specificity was desirable because infection of *I. ricinus* with *B. microti* was a priori assumed to be rare, and underestimation of prevalence therefore is conservative. Regardless, the proportion of ticks that appear to be infected by *B. microti* is similar to that in coastal New England (S. Telford, unpub. data). Recently, Duh et al. reported a similarly high prevalence of *B. microti* infection in nymphal *I. ricinus* ticks collected in Slovenia (7 of 69 ticks tested by PCR) (17). In combination with our findings, this report suggests that *B. microti* infection in *I. ricinus* ticks is far more common than traditionally thought. In addition, vector competence of *I. ricinus* for *B. microti* has been demonstrated experimentally (7,18). The frequent infection of *I. ricinus* with this piroplasm therefore implies zoonotic relevance of this vector-pathogen association in Switzerland and possibly in other parts of Europe.

B. microti transmission is clustered in the study site. Similar to tick-borne encephalitis virus, *B. microti* seems to be maintained in small focal areas. The risk of human infection therefore is spatially highly variable and conditional on tick density. Preliminary analysis of tick infection data from the study site over a 3-year period (Foppa, unpub. data) suggests that *B. microti* is locally maintained, especially in the lower portion of that site, while the exact location of the maximum risk changes over the years.

Residents of this region in eastern Switzerland appear to be exposed to bites by ticks infected with *B. microti*. The serologic result is unlikely to reflect low specificity of the assay, as previous evaluation of this IFA has demonstrated high specificity (19,20). As part of this evaluation, we tested 50 sera from residents of Iceland, where ticks capable of transmitting *B. microti* are absent; none of the sera reacted with *B. microti* antigen (19). We recently repeated testing of these sera and obtained similar results. This finding suggests a satisfactory positive predictive value of the serologic test even in settings of low prevalence.

In the northeastern United States, *B. microti* seroprevalence has varied in endemic regions, from 3.7% in Red Cross blood donors on Cape Cod, Massachusetts (21), to 2.5% and 9.5% in Connecticut residents who were seronegative and seroreactive, respectively, to *Borrelia burgdorferi* (22). Our findings of high local prevalence of *B. microti* infection in *I. ricinus* ticks may seem counterintuitive given the lower seroprevalence in residents of our study area. The findings may, however, reflect a high degree of spatial clustering of transmission with a low average risk (18). Alternatively, the low seroprevalence may be the result of low test sensitivity resulting from antigenic differences between North American *B. microti* strains, which were used for the serologic testing, and the European strains to which our study population had been exposed.

At least locally, the potential for zoonotic transmission of *B. microti* by *I. ricinus* is considerable, which explains serologic evidence in human beings of exposure to this agent in parts of Europe. The lack of recognized human pathology associated with European strains of *B. microti*, despite exposure to infectious tick bites, may be a consequence of a lower virulence of European strains than those of North American. Disease episodes due to *B. microti*, on the other hand, may be overlooked because of the relative nonspecificity of signs and symptoms and the presumption that this agent rarely infects *I. ricinus*.

RESEARCH

Dr. Foppa is assistant professor of epidemiology at the Norman J. Arnold School of Public Health, University of South Carolina, Columbia. His main interests include quantitative methods in infectious disease epidemiology and dynamics of vector-borne zoonoses.

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Address for correspondence: Ivo M. Foppa, Department of Epidemiology and Biostatistics, Norman J. Arnold School of Public Health, 800 Sumter Street, Columbia, SC 29208, USA; fax: 803-777-2524; e-mail: ifoppa@sc.edu

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Rickettsialpox in North Carolina: A Case Report

**Allan Krusell,* James A. Comer,†
and Daniel J. Sexton‡**

We report a case of rickettsialpox from North Carolina confirmed by serologic testing. To our knowledge, this case is the first to be reported from this region of the United States. Including rickettsialpox in the evaluation of patients with eschars or vesicular rashes is likely to extend the recognized geographic distribution of *Rickettsia akari*, the etiologic agent of this disease.

R ickettsialpox is caused by infection with *Rickettsia akari*. Disease in humans was first described in 1946 in residents of apartments clustered in a three-block area in the borough of Queens, New York City (1). Subsequently, small outbreaks of rickettsialpox were recognized in several U.S. cities, including Boston, Cleveland, Philadelphia, Pittsburgh, and West Hartford (2). Most cases to date have occurred in large metropolitan areas of the northeastern United States; about half the described cases have occurred in New York City. However, rickettsialpox is likely more common in the United States than suggested by the relatively small number of reported cases during the past 50 years (3,4).

R. akari is transmitted among house mice (*Mus musculus*) and to humans by the house mouse mite (*Liponyssoides sanguineus*) (2). Recently, cases of rickettsialpox have been reported in residents of the Ukraine (5) and Croatia (6). Isolations have also been made from Korean voles in an area where rickettsialpox has not been reported (7). These data suggest that silent sylvan cycles of *R. akari* infection exist and that the organism is more widely distributed than currently appreciated. To our knowledge, rickettsialpox has not previously been reported in patients in the southern United States. We describe a recently diagnosed *R. akari* infection in a man who resides in a suburban area of North Carolina.

Case Report

A 48-year-old man who worked at a golf course was admitted to a North Carolina hospital with fevers, chills, headaches, and a rash. Seven days before admission, he noted discomfort on the back of his right calf. The patient stated that he thought something had bitten him at this site, although he had not seen any insects or ticks. Over the next 2 days, thigh tenderness and a papule developed at the site of the original discomfort. Three days before admission, the papule began to

*Northeast Medical Center, Concord, North Carolina, USA; †Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and ‡Duke University Medical Center, Durham, North Carolina, USA

ulcerate, and fevers, chills, headaches, and general malaise were present. Two days before admission, several red macules appeared over the anterior chest. Over the next 24 hours, vesicles appeared near the center of these macules.

The patient had a pet dog and cat and had not traveled outside North Carolina in the 3 months before admission. He reported that he had no known exposures to ticks or recent tick bites. He was unaware of any rodents in his house or any local rodent extermination projects. However, he recalled that a stray cat periodically brought dead mice to the general area where he worked, although he never directly touched them.

On admission, the patient appeared ill and was febrile. An eschar was present on his posterior right lower leg (Figure 1). Approximately 30 erythematous macules were noted on his trunk, arms, and legs (Figures 2 and 3). Many of these macules had small central vesicles. Laboratory testing showed normal values for electrolytes and creatinine, hematocrit, and leukocytes. His platelet count was $85,000/mm^3$. Routine blood cultures were sterile.

A diagnosis of rickettsialpox was made, and therapy was started with doxycycline and cefazolin. After 48 hours, the patient became afebrile, and his constitutional symptoms abated. He was discharged, took doxycycline orally for an additional 7 days, and recovered completely.

Two serum samples were submitted to the Centers for Disease Control and Prevention for confirmatory testing. Samples were tested by a standard immunofluorescent antibody assay (IFA) for immunoglobulin G antibodies reactive with *R. akari* and *R. rickettsii* antigens. Because of cross-reactivity among the spotted fever group rickettsiae, confirmatory cross-adsorption testing was done as described (8). Higher reciprocal titers were obtained to *R. akari* antigens than to *R. rickettsii* antigens in both samples (reciprocal titers of 1,024 versus 512 on August 29 and 512 versus 256 on October 11, respectively). Adsorption with *R. akari* greatly reduced titers to both antigens (<16 to both antigens for both samples), whereas adsorption with *R. rickettsii* only partially lowered titers to both antigens (512 versus 256 for the first sample; 128 versus 64 for the

Figure 1. Eschar on posterior right calf of patient with rickettsialpox.

Figure 2. Multiple papulovesicles involving the upper trunk on a patient with rickettsialpox.

Figure 3. Closer view of papulovesicular lesions on patient with rickett-sialpox.

second sample). This pattern of differential reduction in titers is confirmatory for a serologic diagnosis of rickettsialpox (9).

Conclusions

This patient's illness was typical of rickettsialpox. He had a classic eschar, and his vesicular rash, severe headache, and thrombocytopenia are characteristic findings of infection with *R. akari*. However, not all patients with rickettsialpox have a vesicular rash. Although isolation of *R. akari* was not attempted and a skin biopsy was not performed, the results of the IFA testing confirmed the diagnosis.

Rickettsialpox may have occurred sporadically in North Carolina in the past, but the incidence of this disease in this state is probably extremely low. As Figures 1–3 illustrate, infection with *R. akari* produces unusual and characteristic skin abnormalities. Because cases of rickettsialpox may be confused with chickenpox or other viral exanthematous diseases, misdiagnosis may occur when sporadic cases occur in areas where the disease is unknown to local practitioners. The vesicular rash of rickettsialpox may be easily confused with

the skin rash seen in patients with chickenpox; however, the presence of one or more eschars at the site(s) of inoculation, the lack of successive crops of vesicles over time, and the presence of thrombocytopenia should lead clinicians to exclude varicella-zoster virus (formal name: *Human herpesvirus 3*) as the etiologic agent.

In large cities, *R. akari* is maintained in a cycle that includes the house mouse and its associated mite (2). Although our patient did not recall direct exposure to rodents, he did recall a stray cat's bringing mice into the work area. The patient may have been exposed to infected mites in this manner, although he may also have been unknowingly exposed to rodents and their mites at some other location.

Evaluation of patients with eschars or vesicular rashes for rickettsialpox is likely to extend the recognized geographic distribution of *Rickettsia akari*, the etiologic agent of this disease.

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Address for correspondence: James A. Comer, Viral and Rickettsial Zoonoses Branch, Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop G13, Atlanta, GA 30333, USA; fax: 404-639-4436; e-mail: jnc0@cdc.gov

Mycobacterium avium subsp. *paratuberculosis* Infection in a Patient with HIV, Germany

**Elvira Richter,* Johannes Wessling,†
Norbert Lügering,† Wolfram Domschke,†
and Sabine Rüsch-Gerdes***

Mycobacterium avium subsp. *paratuberculosis* (MAP), the causative agent of Johne disease in ruminants, has been incriminated as the cause of Crohn disease in humans. We report the first case of human infection with MAP in a patient with HIV; infection was confirmed by obtaining isolates from several different specimen types.

Opportunistic infections caused by various *Mycobacterium* species are among the leading AIDS indicator diseases in HIV-positive patients (1). Infections with nontuberculous mycobacteria occur mainly in patients who have low CD4+ counts (<50 cells) or high virus counts (2); *Mycobacterium avium* complex is the most important mycobacterial species. *M. avium* complex includes the species *M. avium* and *M. intracellulare*, with *M. avium* consisting of *M. avium* subsp. *avium*, *M. avium* subsp. *sylvaticum,* and *M. avium* subsp. *paratuberculosis* (MAP). All these subspecies have identical 16S rRNA gene and 16S to 23S transcribed spacer sequences, as well as shared biochemical characteristics (3). However, MAP is dependent on mycobactin for its growth, whereas *M. avium* grows well on different solid media.

MAP is the causative agent of Johne disease, a chronic granulomatous ileitis occurring mainly in ruminants (4). MAP has been incriminated as the cause of Crohn disease in humans (5,6), although conflicting findings have been reported. However, culture-confirmed cases of MAP in human specimens remain rare (5,6).

Case Report

A 36-year-old HIV-positive man, who had been treated at our hospital since 1995 for HIV, hepatitis C, and hemophilia, had profuse diarrhea (6–8 episodes/day), fever as high as 39.9°C, and 10 kg of body weight loss in 5 weeks. Laboratory findings included hemoglobin 9.6 g/dL, pseudocholinesterase 2,099 U/L, HIV-DNA virus count 500 copies/mL, CD4+ lymphocyte count 29 x 10^6/mL, and C-reactive protein 76 mg/L. Stained colon tissue samples, bone marrow punch, and liver biopsy showed abundant acid-fast bacilli. Endoscopic findings

*National Reference Center for Mycobacteria, Borstel, Germany; and †University of Münster, Münster, Germany

on colonoscopy were multiple polypoid lesions approximately 5 mm in size in the transverse and sigmoid colon.

Microbiologic analyses included culture for mycobacteria (liquid media: BACTEC 460TB or MGIT [Becton, Dickinson and Company, Cockeysville, MD] and solid media produced in-house, all media without supplementation of mycobactin) from at least 21 specimens (blood, urine, sputum, biopsy, feces) over a 3-year period. Of these, eight specimens (blood, feces, and biopsy) were positive for mycobacteria in liquid media after 6 to 16 weeks of incubation. Subcultures remained negative on Löwenstein-Jensen slants but after approximately 4 weeks became positive on mycobactin-supplemented Middlebrook slants with colorless dysgonic colonies. Microscopic examination of these colonies showed acid-fast bacilli (Figure 1).

For species identification, AccuProbe assays (Gen-Probe, San Diego, CA) for *M. avium* complex were performed on liquid media, all yielding strong positive results. However, repeated attempts to perform drug-susceptibility testing in the liquid BACTEC 460TB system were unsuccessful because of insufficient growth of the control. Since *M. avium* complex usually grows very well, the primary identification was questionable. Thus, polymerase chain reaction (PCR) for the amplification of a part of the mycobacterial gene coding for the ribosomal 16S RNA and additional sequencing was performed from two positive cultures (7). The resulting sequence was compared with those stored in the International
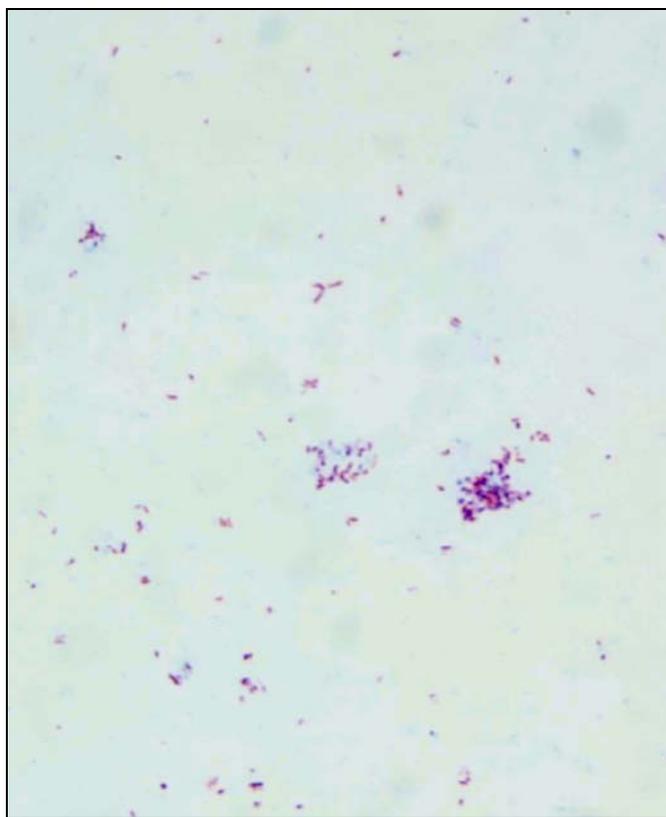

Figure 1. Ziehl-Neelsen–stained micrograph of *Mycobacterium avium* subsp. *paratuberculosis* colonies growing on mycobactin-supplemented Middlebrook agar.

Nucleotide Sequence Database (8), showing the signature sequence of *M. avium*/*M. paratuberculosis*, which is identical for both species and confirmed the AccuProbe results. For further differentiation between *M. avium* and MAP, PCR targeting the insertion sequence IS*900* (primer: IS*900*-1: 5´-TGTTCGGGGCCGTCGCTTAG; IS*900*-2: 5´-CGTTCCAGC GCCGAAAGTAT), which is present only in MAP strains (9), was done with the two most recent positive cultures. This assay showed clearly positive results from the two cultures tested and the MAP type strain, while the *M. avium* strains remained negative (Figure 2).

Because acid-fast bacilli were identified in biopsy specimens, treatment was started with ethambutol, ciprofloxacin, clarithromycin, and rifabutin. Initially, no clinical improvement was observed, and the patient's weight loss and daily fever of 39C°–40°C continued. When ciprofloxacin was replaced with levofloxacin, progression of the infection appeared to stop. However, the patient died from cardiorespiratory failure.

Conclusions

We describe the case of an HIV-infected patient who had a severe mycobacterial disorder thought to be caused by *M. avium* complex. Because growth was insufficient for susceptibility testing, the presence of MAP was assumed; however, the assumption was made after 2 years, because of difficulties in isolating MAP from human specimens (e.g., blood) in media not thought to enable its growth. Finally, the demonstration of the insertion sequence IS*900*, an assay not routinely performed in human diagnostic laboratories like ours, confirmed this hypothesis.
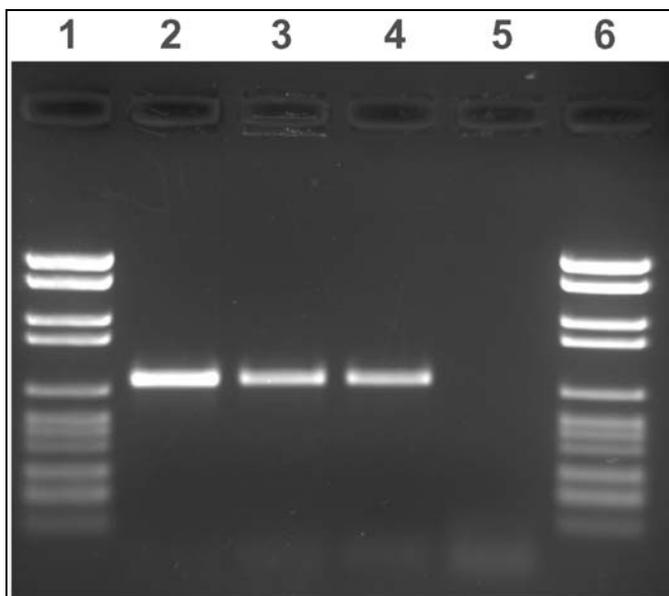

Figure 2. Agarose gel electrophoresis of amplified IS*900* fragments. Lanes 1 and 6: molecular weight marker (2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234–220, 154 bp); lanes 2 and 3: two patient samples; lane 4: positive control (*Mycobacterium avium* subsp. *paratuberculosis* type strain); and lane 5: negative control (*Mycobacterium avium* strain).

MAP isolated from human specimens has not yet been demonstrated by routine techniques. Several studies have reported the presence of MAP DNA in association with Crohn disease, although culture confirmation remains rare in these patients (5,6).

In the case we describe, mycobacterial growth could be detected in liquid media in 8 of 21 specimens, all confirmed as *M. avium* complex/*M. paratuberculosis*. However, because of the limited growth, we assume the presence of MAP even in those specimens not tested by IS*900* PCR. These results indicate that MAP can grow to a limited extent in routine liquid media without mycobactin supplementation, at least if present in high amounts in the specimen.

Susceptibility testing of the isolated strains could not be performed because of insufficient growth. Reports on susceptibility testing of MAP are rare, yet data obtained by a luciferase-based susceptibility assay (10) indicate susceptibility at least to clarithromycin and rifabutin, which were included in therapy. However, the patient's response to treatment was not clearly positive and may have been hampered by his general poor health. This report suggests a pathogenic role of MAP for immunocompromised patients, raising the question of whether this strain so far has not been detected because of its limited growth, whether it has been misidentified as *M. avium*, or whether its occurrence in infections is low. However, herd prevalence of bovine paratuberculosis has been reported to range from 7% to 55% in Europe and to reach approximately 40% in stocks of >300 animals in the United States (4). Thus, consumption of inadequately pasteurized dairy products may be a possible risk for infection, especially for immunocompromised patients.

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Address for correspondence: Elvira Richter, Forschungszentrum Borstel, National Reference Center for Mycobacteria, Parkallee 18, 23845 Borstel, Germany; fax: 49-4537-188311; e-mail: erichter@fz-borstel.de

EMERGING INFECTIOUS DISEASES

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Role of Electronic Data Exchange in an International Outbreak Caused by *Salmonella enterica* Serotype Typhimurium DT204b

Elizabeth A. Lindsay,* Andrew J. Lawson,*
Rachel A. Walker,* Linda R. Ward,* Henry R. Smith,*
Fiona W. Scott,* Sarah J. O'Brien,† Ian S.T. Fisher,†
Paul D. Crook,† Deborah Wilson,‡ Derek J Brown,§
Hjordis Hardardottir,¶ Wim J.B. Wannet,**
Helmut Tschäpe,†† and E. John Threlfall*

From July through September 2000, patients in five European countries were infected with a multidrug-resistant strain of *Salmonella* Typhimurium DT204b. Epidemiologic investigations were facilitated by the transmission of electronic images (Tagged Image Files) of pulsed-field gel electrophoresis profiles. This investigation highlights the importance of standardized protocols for molecular typing in international outbreaks of foodborne disease.

The Study

From July through September 2000, patients in five European countries (England, Scotland, Germany, the Netherlands, and Iceland) were infected with a strain of *Salmonella enterica* serotype Typhimurium definitive phage type (DT) 204b; the strain was resistant to ampicillin (A), chloramphenicol (C), gentamicin (G), kanamycin (K), streptomycin (S), sulphonamides (Su), tetracyclines (T), trimethoprim (Tm), and nalidixic acid (Nx). The strain also had decreased susceptibility to ciprofloxacin (CpL), with an MIC by E-test of 0.38 mg/L (1,2). Over 350 laboratory-confirmed cases were recognized. Epidemiologic investigations implicated shredded lettuce as the vehicle of infection (1).

Isolates from patients in Iceland, the Netherlands, and Scotland were referred to the England and Wales Public Health Laboratory Service (PHLS) Laboratory of Enteric Pathogens for phage typing and antibiogram analysis. These isolates were compared with those from a concurrent outbreak of multiresistant *S.* Typhimurium DT204b in northeastern England and from patients returning to England and Wales after visiting other European countries. A panel of seven isolates from outbreaks in England, Scotland, Iceland, and the Netherlands and from patients returning to the United Kingdom after visiting Greece, Germany, and the Netherlands were further characterized by a variety of molecular techniques, including plasmid profile typing, pulsed-field gel electrophoresis (PFGE), fluorescent amplified fragment-length polymorphism fingerprinting (FAFLP), and integron typing; specific resistance genes were characterized by polymerase chain reaction (PCR) and the mutation conferring decreased susceptibility to ciprofloxacin was identified by a LightCycler (Roche Diagnostics Ltd., Lewes, U.K.) *gyrA* mutation assay (GAMA) (3). Isolates from Germany and Scotland were typed independently with the same phenotypic methods as those used in the Laboratory of Enteric Pathogens and also by plasmid profile typing and PFGE. To facilitate epidemiologic investigations, PFGE Tagged Image Files (TIFs) of banding patterns of isolates from England, Scotland, and Germany were exchanged electronically.

Conjugation experiments were performed at both 28°C and 37°C by using a rifampicin-resistant strain of *Escherichia coli* (strain 20R764) as the recipient. Resultant plasmids were classified by incompatibility. DNA was extracted from transconjugants by using a DNeasy tissue kit (Qiagen Ltd., Crawley, U.K.). For plasmid profile analysis, plasmids were resolved by electrophoresis at 110 V for 3 hours in 0.8% wt/vol agarose. Oligonucleotide primers synthesized by MWG-Biotech UK Ltd. (Milton Keynes, U.K.) were used to detect the antibiotic resistance genes *aad*A2, bla_{CARB-2}, bla_{TEM}, *sul1*, *tet*A (class A), *tet*A (class G), *tet*A (class B), and integrons in both wild-type strains and *E. coli* K12 transconjugants. The nucleotide sequence of these primers and the corresponding temperature profiles for amplification have been described (4). GAMA, designed to detect three different *gyrA* mutations, was performed in a LightCycler instrument under previously described reaction components and conditions (3). For PFGE, agarose plugs were prepared by the method of Powell et al. (5) with 2% chromosomal grade agarose (Bio-Rad Laboratories, Hemel Hempstead, U.K.) replacing the 2% Type VII LGT agarose (Sigma Chemical Co., Poole, U.K.). Samples were run through a 1% pulsed-field certified agarose gel (Bio-Rad) at 180 V for 44 hours, with pulse times ramped from 6 to 72 seconds. For FAFLP, the selective primer combinations *Eco*+0 *Mse*+T, *Eco*+0 *Mse*+TA, and *Eco*+0 *Mse*+CG were used, and gel separation and fragment analyses were performed, all as described by Scott et al. (6).

All seven isolates had phage typing reactions corresponding to *S.* Typhimurium DT204b. This rare phage type was reported in only 40 cases in England and Wales in the 5-year period 1996–2000 and had never been identified in Iceland before this epidemic.

*Public Health Laboratory Service Laboratory of Enteric Pathogens, London, United Kingdom; †Public Health Laboratory Service Communicable Diseases Surveillance Centre, London, United Kingdom; ‡County Durham and Darlington Health Authority, Durham, United Kingdom; §North Glasgow University Hospitals National Health Service Trust, Glasgow, Scotland; ¶Landspitali University Hospital, Reykjavik, Iceland; **National Institute of Public Health and the Environment, Bilthoven, the Netherlands; and ††Robert-Koch Institut, Harz, Germany

With the exception of resistance to nalidixic acid and decreased susceptibility to ciprofloxacin, the complete resistance spectrum (ACGKSSuTTm) was transferable to *E. coli* K12 rif^r as an intact linkage group. Plasmid profile analysis demonstrated the presence of five plasmids of 120, 65, 4.0, 3.0, and 2.0 MDa. Most transconjugants with the resistance pattern ACGKSSuTTm had a single incompatibility group H$_2$ plasmid of 120 MDa. A few transconjugants had both the 120-MDa plasmid and an additional plasmid of 65 MDa. When PCR amplification was performed on DNA from transconjugants harboring either the single 120-MDa plasmid or both the 120-MDa and 65-MDa plasmids, positive results for *aad*A2, *bla*$_{TEM}$, *sul1,* and *tet*A (class A) were obtained; *bla*$_{carb-2}$, *tet*A (class G), and *tet*A (class B) were negative. When studied by integron PCR, one discrete 1.6-kb band was generated in transconjugants when either the 120-MDa plasmid or both the 120-MDa and the 65-MDa plasmids were present. In addition, one very faint amplicon of approximately 4 kb was consistently produced. Whether both the 120-Mda plasmid and the 65 Mda-plasmid have tetracycline resistance genes or whether tetracycline resistance is encoded by only the 120-MDa plasmid is unclear. When studied by GAMA, the *gyrA* mutation was that of aspartate to glycine (G**A**C-G**G**C) at codon 87.

PFGE profiles of all seven isolates were indistinguishable (Figure). To facilitate epidemiologic investigations, PFGE and plasmid profile TIFs of the banding patterns of these isolates and those of multiresistant *S.* Typhimurium DT204b from Scotland and Germany were exchanged electronically. In all cases, the resultant PFGE and plasmid profiles were indistinguishable. Finally, when studied by FAFLP, the seven isolates were identical.

Conclusions

These results confirm that the strains of *S.* Typhimurium DT204b of R-type ACGKSSuTTmNxCp$_L$ responsible for outbreaks of infection in five European countries in the summer of 2000 were indistinguishable by all phenotypic and molecular criteria used for their characterization. A key aspect of this investigation was the rapid exchange of molecular fingerprints between laboratories already using standardized phage typing and antimicrobial susceptibility testing. In the United States, the exchange of molecular data has been addressed by the establishment of PulseNet, a national molecular typing scheme based on a standard method for PFGE; a similar network is being set up for the major Salmonella reference laboratories in Europe with research funding from the European Commission. Developing compatible networks for the exchange of real-time molecular data for *S. enterica* on an intercontinental scale would be of major benefit for the global control of salmonellosis.

Ms. Lindsay is a research scientist completing a doctoral degree in studies on antibiotic resistance in organisms from farm wastes. Her primary research interests are in the molecular epidemiology of antibiotic resistance in gram-negative bacteria.


Lanes 1& 9: 48.5-kb ladder;
Lane 2: P5283600 (England)
Lane 3: P5326480 (Scotland)
Lane 4: P5304530 (Iceland)
Lane 5: P5310250 (Netherlands)
Lane 6: P5283670 (Greece)
Lane 7: P5285060 (Germany)
Lane 8: P5294700 (Netherlands)
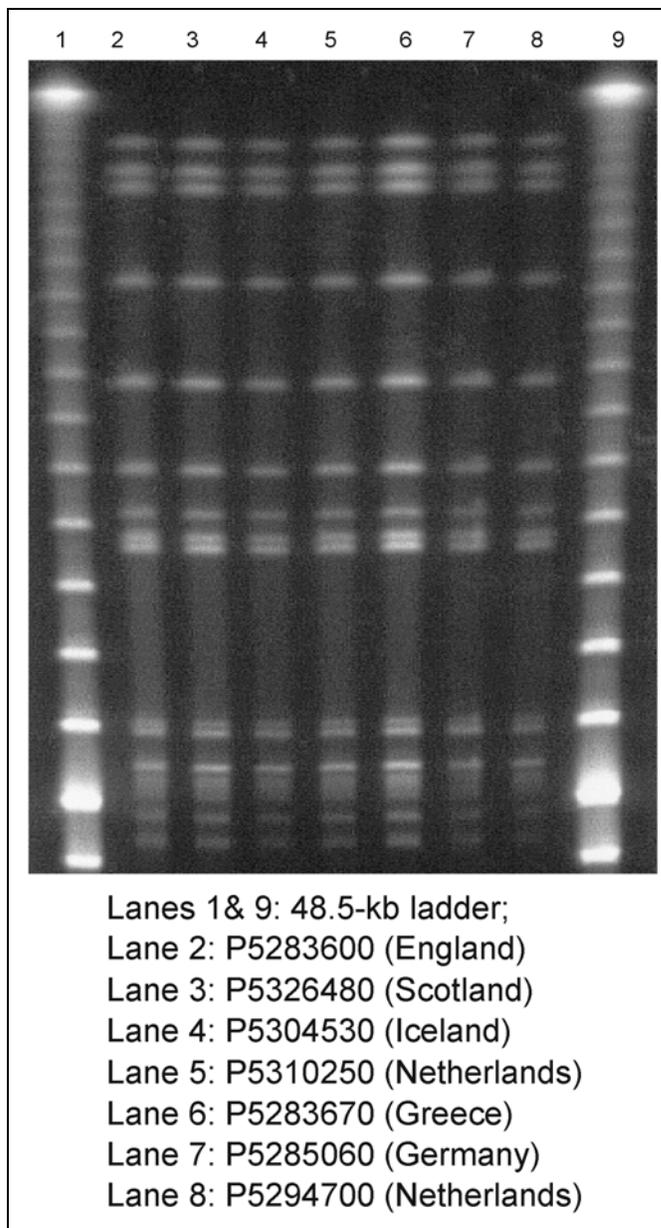
Figure. Pulsed-field gel electrophoresis profiles of *Xba*I-digested genomic DNA from isolates of *Salmonella enterica* serotype Typhimurium DT204b.

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Address for correspondence: E. John Threlfall, Public Health Laboratory Service Laboratory of Enteric Pathogens, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT, United Kingdom; fax: 44 (0)208 905 9929; e-mail: jthrelfall@phls.org.uk

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Novel Measles Virus Genotype, East Timor and Australia

Doris Chibo,* Michaela Riddell,* Michael Catton,* and Christopher Birch*

Measles outbreaks in 1999 in Queensland and Victoria, Australia, were caused by a novel strain of clade G virus (proposed name g3). Epidemiologic and molecular evidence supports independent circulation of this virus in Queensland, northern Australia, in addition to importation of the virus by East Timor refugees seeking safe haven in Australia.

S trategies to reduce *Measles virus* (MeV) circulation in industrialized countries, such as aggressive vaccination campaigns targeting children, have proven successful (1). Nevertheless, through introduction from other geographic locations, the virus continues to cause outbreaks in industrialized countries in unvaccinated persons.

In Victoria, measles is a reportable disease, and an enhanced surveillance strategy has been operational since 1997. A registered nurse visits the homes of patients with reported cases to collect specimens for laboratory confirmation, by polymerase chain reaction or detection of MeV-specific immunoglobulin (Ig) M, of the clinical diagnosis and subsequent identification of the measles genotype (2). For other Australian states, specimens from laboratory-confirmed cases are sent for genotyping to the WHO regional measles reference laboratory for the western Pacific Region.

Variable regions in the MeV genome include the hemagglutinin (H) and nucleoprotein (N) genes. The most variable region is the carboxyl-terminal end (450 nucleotides [nt]) of the N gene. A uniform nomenclature approved by World Health Organization (WHO) has existed since 1998 and is used in classifying and naming measles viruses. Currently, 20 genotypes and 1 proposed new genotype exist, encompassing eight clades designated A–H. Each clade contains MeV genotypes that are related by ≥2.5% nt divergence in the 450-bp carboxyl-terminal end of the N gene and 2% in the H gene (3). Clades are distinguished by greater nucleotide differences, location of nucleotides, and specific nucleotides shared in genotypes of a particular clade.

Until recently, MeV strains belonging to clade G had not been detected for >15 years, and the lineage was considered to be either extinct or inactive (4). However, retrospective sequence analysis of a measles strain isolated from an immunocompromised infant from the Netherlands, who had been infected in Indonesia in 1997, and of measles strains associated with outbreaks in Indonesia and Malaysia in 1999 have

demonstrated that this genotype has circulated in the intervening period (5–6). We describe the circulation of a novel genotype of MeV in Australia and investigate its likely origin.

The Study

In June 1999, the novel genotype was first identified in Victoria in a 24-year-old Australian man who had symptoms of MeV infection shortly after visiting the northern state of Queensland. In the next 2 months, four more cases of infection with the same genotype were identified in Queensland. Epidemiologic links between these five cases could not be established.

In the second week of September 1999, refugees from the newly independent country of East Timor arrived in Darwin, Northern Territory, where they underwent preliminary medical examinations before being moved to a safe-haven refugee camp in regional Victoria. Several days after arriving at the safe haven, a 4-year-old girl had symptoms that matched the clinical case definition of measles infection (rash and cough with fever at onset of rash) (7). The diagnosis was confirmed serologically with an Enzygnost Anti-measles-virus/IgM kit (Dade Behring, Marburg, Germany). Active surveillance of all contacts of the initial patient and all residents reporting to the safe-haven medical center with symptoms suggestive of measles infection identified 11 other cases (4 laboratory confirmed) in children <13 years of age in the safe haven; a 26-year-old volunteer worker who worked at the accident and emergency department of the same hospital that admitted some of the infected refugees was also diagnosed with measles infection. Subsequent molecular analysis confirmed the volunteer's infection as being caused by the same virus as the refugees. The last recognized case of this MeV genotype occurred in early November 1999, when a United Nations International Force East Timor (Interfet) soldier, who showed clinical symptoms in East Timor immediately before he was transferred to Darwin, was confirmed as being infected with MeV.

The commonality of the virus strains circulating in both Australia and East Timor was confirmed through analysis of MeV RNA (8). Analysis of the 456-bp carboxyl-terminal region of the N gene of the Victorian and Queensland samples yielded identical sequences in most cases, suggesting that these patients were infected with the same strain of MeV as the East Timorese. Nevertheless, no epidemiologic links were apparent between the Queensland cases and the subsequent cases in East Timorese refugees.

The N gene sequences of measles viruses identified from June to November 1999 were most closely related to the Amsterdam prototype G2 strain identified by de Swart et al. (4). Although phylogenetic analysis indicated that these sequences belonged within the clade G viruses, they differed from the G2 prototype strain by 12 (2.6%) nt and by deduction 6 (4%) amino acids (Figure). N gene sequences were obtained from 17 clinical samples. As a group, these 17 samples shared an amino acid (439K) in the N protein not previously seen in any other reference prototypes analyzed. Thirteen of these

*World Health Organization Regional Measles Reference Laboratory for the Western Pacific Region, North Melbourne, Victoria, Australia
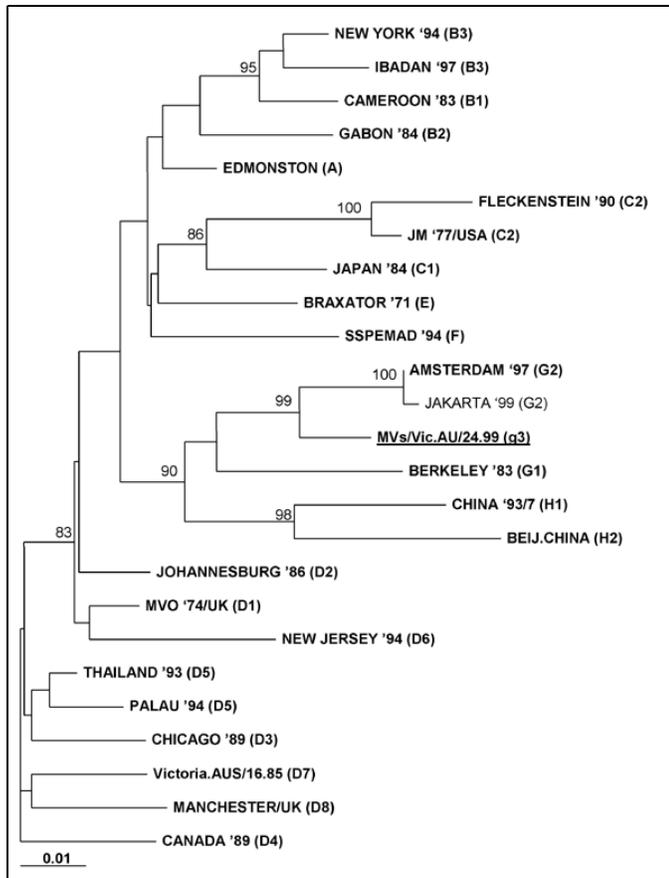
Figure. Phylogenetic analysis with the Phylip software program of DNA-dist (maximum likelihood/neighbor-joining, 1000 bootstrap cycles) of the carboxyl-terminal 456-bp nucleoprotein (N) gene sequence of measles virus/Vic.AU/24.99 circulating in Australia and East Timor. World Health Organization–designated prototype strains are shown in bold, and the proposed new g3 genotype is shown in bold and underlined. Jakarta 1999 (G2) has also been included to show the difference between the clade G viruses. Statistically significant bootstrap values (>80%) are indicated. Scale (0.01) indicates nucleotide substitutions per site.

Table. Unique predicted amino acid differences compared with all reference measles virus sequences in 456-bp carboxyl-terminal end of N[a] gene[b]

Sample no.	Predicted amino acid differences	Accession no.
1–13	439K[c]	AF353622
14–16	439K[c]; 456L	AY055850
17	439K[c]; 456L	AY055851

[a]N, nucleoprotein.
[b]Identified from 17 clinical samples in the outbreak.
[c]Unique to proposed genotype g3.

sequence also contained amino acids in the H protein not previously found in other reference prototypes, namely 212Q, 225H, 238D, and 495N. Phylogenetic analysis of the full H gene sequence showed similar patterns of relatedness to those obtained for the N gene (results not shown).

Together, phylogenetic analysis of the N and H genes and the appearance of novel amino acids in the H protein provided strong evidence that these measles virus were sufficiently unrelated to the prototype G2 strain to enable their classification as a new genotype within clade G. We propose that measles virus/Vic.AU/24.99 be the reference sequence for a new genotype, g3, pending isolation of a reference strain (3). GenBank accession numbers for the 456-bp carboxyl-terminal end of three of the N gene sequences are shown in the Table. The GenBank accession number for the full-length H gene is AF35362.

The novel g3 MeV was not the only strain circulating in Australia during this time. Coinciding with the genotype g3 outbreak, four cases of genotype D8 were identified in Victoria. Three months earlier, small clusters of measles cases were identified in two other Australian states, genotype D5 in the Northern Territory and genotype D3 in Western Australia. Soon after the g3 outbreak, genotype D7 circulated in Victoria, Queensland, and the Northern Territory.

In recent years, vaccination campaigns have been undertaken in East Timor under the guidance of United Nations Children's Fund (UNICEF) and WHO. In October 1999, UNICEF conducted a major immunization campaign in Dili, East Timor, reaching approximately 4,000 children <5 years of age; in March 2000, >45,000 children were vaccinated (9). Nevertheless, from January 2000 to May 2001, a total of 1,479 cases of suspected measles infection were reported in East Timor.

In an industrialized country like Australia, where no apparent circulation of an indigenous MeV strain occurs, the use of epidemiologic surveillance and molecular characterization is important in tracing the source and transmission pathways of MeV imported from areas where the disease is endemic. Apart from expanding our global knowledge of MeV genotypes, molecular characterization is also useful in clarifying epidemiologic links and distinguishing between vaccine-associated and wild-type infection.

Despite the novelty of the circulating MeV genotypes, our findings highlight the need for continued vigilance if the virus

samples were identical in sequence (samples 1–13, Table). Three samples (samples 14–16, Table) differed by a single nucleotide, which resulted in another novel amino acid change, P456L. One sample (sample 17, Table) diverged by 2 nt, one silent and the other resulting in the same P456L change seen in samples 14–16. Differences seen in these 17 samples did not appear to be related to the geographic location of the cases.

Full or partial H gene sequences were derived from the measles viruses of four samples obtained from patients 8, 13, 15, and 16. Phylogenetic analysis of the H gene of the first 842 nt of these samples confirmed their assignment to the clade G viruses (results not shown). The four sequences analyzed showed up to 1.1% intranucleotide divergence. The MeV from patient 8 varied by 2.1% nt sequence compared with the prototype G2 strain. Comparison of nucleotide and amino acid sequence of the H gene of the one sample (from patient 13) with full H gene sequence showed differences from the prototype G2 strain of 35 (1.9%) nt and 13 (2.1%) amino acids. This

is to be eradicated. The humanitarian movement of refugees from a country where MeV infection is uncontrolled to countries with relatively high MeV herd immunity is now a common occurrence. Thus, the potential for transmission of this highly infectious virus to residual susceptibles in the wider community remains a distinct possibility, as demonstrated by this outbreak. Measures, such as vaccination of military personnel and support staff working directly with displaced persons; use of appropriate infection-control procedures when attending sick refugees; and screening of newly arrived refugees from measles-endemic areas, are likely to decrease transmission of MeV in industrialized countries.

Acknowledgments

Ms. Chibo is a medical scientist at the Victorian Infectious Diseases Reference Laboratory in Victoria, Australia. Her main interests are the use of molecular techniques and phylogenetic analysis to study viruses of public health importance and mechanisms associated with development of resistance to antiviral drugs by herpesviruses.

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Address for correspondence: Christopher Birch, 10 Wreckyn St., North Melbourne 3051, Victoria, Australia; fax: 61 3 9342 2666; e-mail: chris.birch@mh.org.au

First Outbreak of Dengue Hemorrhagic Fever, Bangladesh

**Mahbubur Rahman,* Khalilur Rahman,†
A. K. Siddque,* Shereen Shoma,* A. H. M. Kamal,†
K. S. Ali,* Ananda Nisaluk,‡ and Robert F. Breiman***

During the first countrywide outbreak of dengue hemorrhagic fever in Bangladesh, we conducted surveillance for dengue at a hospital in Dhaka. Of 176 patients, primarily adults, found positive for dengue, 60.2% had dengue fever, 39.2% dengue hemorrhagic fever, and 0.6% dengue shock syndrome. The Dengue virus 3 serotype was detected in eight patients.

D engue fever (DF) and dengue hemorrhagic fever (DHF) are caused by four antigenically distinct but related dengue virus (official name: *Dengue virus* [DENV]) serotypes transmitted primarily by *Aedes aegypti* (yellow fever mosquito). DHF, the severe form of the disease, is endemic and frequently intensifies into epidemics in Southeast Asia, resulting in frequent hospitalizations and deaths (1,2). Recently, dengue has emerged as a substantial global health problem with increased incidence in new countries and tropical areas (3,4). DF was documented in Bangladesh from the mid-1960s to the mid-1990s, but an outbreak of DHF has not been previously reported (5,6). During late June 2000, a 28-year-old patient was admitted to a hospital in Dhaka, Bangladesh, with hemorrhagic fever, ascites, pleural effusion, and thrombocytopenia. An enzyme-linked immunosorbent assay (ELISA) for anti-dengue antibodies confirmed the case as DHF (1). That summer, an outbreak of DF (>5,000 hospitalized cases reported) and DHF occurred in Dhaka and other major cities of Bangladesh (7).

The Study

We began surveillance for dengue among patients at a hospital in Dhaka during July 1–October 31, 2000. Clinical details of each patient were recorded on a standardized form; when indicated, chest radiographs and abdominal ultrasounds were done, in addition to hemoglobin, hematocrit, and total blood and platelet counts. Sera from all patients were tested by ELISA for anti-dengue and anti-Japanese encephalitis viral immunoglobulin (Ig)M and IgG (8). Paired sera were tested when available. Sera from 30 patients with fever of <6 days' duration were also tested for serotype-specific dengue viral RNA by reverse transcription-polymerase chain reaction (RT-PCR) (9). Samples having ≥40 units of IgM or IgG antibodies were considered positive for dengue infection. A ration of IgM

*ICDDR,B: Centre for Health and Population Research, Dhaka, Bangladesh; †Holy Family Hospital, Dhaka, Bangladesh; and ‡The Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand

and IgG <1.8 defined a secondary infection and ≥1.8 a primary infection (8). DF, DHF, and dengue shock syndrome (DSS) were defined according to World Health Organization (WHO) criteria (1) and confirmed by positive IgM or IgG ELISA or RT-PCR. Statistical analysis was done by chi-square test.

Of 336 suspected dengue patients, sera were available for 240 patients; 176 (73.3%) had confirmed dengue infection (168 by ELISA; 2 by RT-PCR; and 6 by both tests). RT-PCR detected dengue virus 3 serotype (DENV-3) in 8 (26.6%) of 30 patients tested. Both acute- and convalescent-phase sera were positive in 18 of 30 paired specimens available, and seroconversion occurred in 2 of the remaining 12 sera. All sera were negative for antibodies to *Japanese encephalitis virus*. Thirty-one (18%) cases were in children (<18 years of age). The highest proportion of cases occurred in persons 18–33 years of age (Figure). DF occurred most commonly (60.2%), followed by DHF (39.2%), and DSS (0.6%). Most (80%) patients reported to the hospital after 5 days of fever. Spontaneous bleeding occurred in 91% of patients with DHF, compared with 25% patients with DF (p<0.01). Frequent clinical features were fever (100%), headache (91%), myalgia/arthralgia (85%), vomiting (64%), macular rash (55%), bleeding (46%) (including melena [20%] and bleeding gums [11.6%]), thrombocytopenia (≤100,000/mL, 56.7%), pleural effusion (12%), ascites (9%), and hepatomegaly (7.5%). Secondary infection was detected in 71% of 174 ELISA-positive cases, more commonly in patients with DHF than with DF (relative risk 2.14, p=0.002) (Table).

All but two adult patients recovered (case-fatality rate 1.14%). One DF patient died with severe hematemesis and melena; another DSS patient died within 2 hours of hospitalization.

Conclusions

Dengue causes more illness and death than any other arboviral infection in the world (4). This first outbreak highlights the geographic expansion of DHF in Bangladesh, where classic DF caused by multiple serotypes had been previously reported (5,6). The DHF outbreak started in late June 2000,
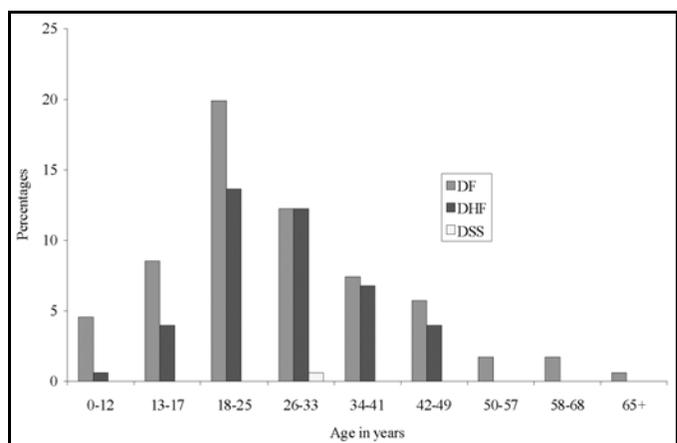

Figure. Age distribution of dengue cases, Bangladesh, 2000.

Table. Distribution of serologically positive dengue cases by type of infections in adults and children, Bangladesh, 2000

Type of infection, by age group[a]	No. (%) of dengue cases, by category			
	Dengue fever (n=104)	Dengue hemorrhagic fever (n=69)	Dengue shock syndrome (n=1)	Total cases (n=174)[b]
Primary	39 (37.5)	11 (15.9)	0 (0)	50 (28.7)
Adults	26 (25.0)	10 (14.5)	0 (0)	36 (20.7)
Children	13 (12.5)	1 (1.4)	0 (0)	14 (8.0)
Secondary	65 (62.5)	58 (84.1)	1 (100)	124 (71.3)
Adults	55 (52.9)	51 (73.9)	1 (100)	107 (61.5)
Children	10 (9.6)	7 (10.1)	0 (0)	17 (9.8)

[a]Adults ≥18 yrs of age; children <18 yrs of age.
[b]Two cases that were negative by enzyme-linked immunosorbent assay and positive by reverse transcription-polymerase chain reaction were not included.

peaked in September (during the rainy season), and subsided in the dry winter season in December 2000. While dengue affected all age groups, adults predominated in this hospital-monitored study. In Singapore, India, Malaysia, and Brazil, where dengue has been epidemic for several years, the mean age of dengue infection is increasing and adults are frequently infected, indicating an epidemiologic change in dengue infection in those locations (1,10).

The precise magnitude of this countrywide outbreak is unknown; 5,575 hospitalized dengue cases were reported to the Ministry of Health in Bangladesh, with a case-fatality rate of 1.61% through mid-November 2000 (6). Most patients had DF, 25% with bleeding manifestations (a severe form of the illness) (1,4). WHO classification of dengue diseases is often not feasible in many countries because of lack of trained health professionals, adequate laboratories, and radiologic support. The facilities to detect DHF by using hematocrit (capillary method) and plasma leakage signs (chest radiograph or ultrasound) are not readily available in many tropical countries. Successful treatment of dengue depends on symptom recognition and careful fluid management (1). Thus, a simple classification scheme of dengue diseases based on symptoms and signs is needed to improve case management and reduce deaths.

DHF is believed to occur as a result of antibody-dependent enhancement of heterotypic-secondary dengue infections (1,4,11). Our findings support the role of sequential infection in the development of DHF. However, occurrence of DHF in some patients with primary infection suggests additional host and virologic factors.

In our study, DSS was a rare event, resulting in a lower case-fatality rate for dengue than reported elsewhere (7), likely representing hospitalization of less severe cases. We have insufficient data to comment on the virulence of the outbreak strain. High numbers of *Ae. aegypti* were identified throughout the city (Y. Wagatsuma, ICDDR,B; pers. comm.). Insecticide spraying, public health education (including community source reduction), and perhaps most importantly, the onset of the dry winter season may have contributed to ending this dengue outbreak in 2000.

Therapeutic strategies proposed by WHO have been widely circulated by the Bangladesh government, and physi-

cians continue to gain experience in proper management of dengue. Ongoing surveillance, vector surveys, and epidemiologic studies to identify risk factors will provide key information for controlling dengue. Ultimately, a safe and effective vaccine will be needed to address this emerging problem in Bangladesh and elsewhere.

Dr. Rahman is a physician, clinical microbiologist, and molecular biologist in Laboratory Sciences Division, ICDDR,B: Centre for Health and Population Research, Bangladesh. His professional interests include infectious diseases, epidemiology, diagnosis, and management of emerging infectious diseases and antimicrobial resistance.

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Address for correspondence: Mahbubur Rahman, Laboratory Sciences Division, ICDDR,B: Centre for Health and Population Research, GPO Box 128, Dhaka 1000, Bangladesh; fax: 880-2-8812529; e-mail: mahbubur@icddrb.org

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West Nile Virus

Detection of West Nile Virus in Oral and Cloacal Swabs Collected from Bird Carcasses

Nicholas Komar,* Robert Lanciotti,* Richard Bowen,† Stanley Langevin,* and Michel Bunning*‡

We evaluated if postmortem cloacal and oral swabs could replace brain tissue as a specimen for West Nile virus (WNV) detection. WNV was detected in all three specimen types from 20 dead crows and jays with an average of >10^5 WNV PFU in each. These findings suggest that testing cloacal or oral swabs might be a low-resource approach to detect WNV in dead birds.

S ince 1999, surveillance of bird deaths has become a standard epidemiologic method for detecting the spread and continued presence of West Nile virus (formal name: *West Nile virus* [WNV]) transmission throughout the eastern United States (1). In 2000 alone, approximately 13,000 bird carcasses were tested for WNV (2). Substantial resources are required to accomplish the tasks associated with this novel type of arbovirus surveillance: transport of the avian carcasses to a laboratory (often distinct from the microbiology laboratory where diagnostic testing will be performed), organ removal, tissue maceration and clarification, and testing of tissue homogenates. We considered ways to simplify these tasks.

Given that birds with acute WNV infection frequently shed the virus in cloacal or oral cavities (3–6) and that we have detected very high WNV titers (e.g. 10^6 PFU) on cloacal and oral (nasopharyngeal) swabs of corvid[1] and other passerine birds with experimentally induced, acute WNV infections (N. Komar, unpub. data), we hypothesized that cloacal swabs or oral swabs from carcasses could replace brain samples, the preferred tissues to test for WNV infection in corvid carcasses (7).

The Study

We collected postmortem specimens from 20 corvids, including 12 American Crows (*Corvus brachyrhynchos*), 4 Fish Crows (*Corvus ossifragus*), and 4 Blue Jays (*Cyanocitta cristata*), that had died (or in one case had been euthanized after becoming moribund) after experimental infection with the New York 1999 strain of WNV. (The modes of infection,[2] sampling protocol, and resulting pathogenesis will be described separately.) Brain and other organs were harvested,

and postmortem cloacal and oral swabs were collected (using standard cotton- or Dacron-tipped applicators) in 0.5-mL physiological buffer containing antibiotics, within 24 hours of death. All specimens were frozen at -70°C until assayed for virus content by Vero plaque assay and for WNV-specific RNA by TaqMan reverse transcriptase–polymerase chain reaction (RT-PCR), as previously described (8).

We detected WNV RNA in all postmortem brain tissue samples as well as cloacal and oral swabs. Infectious WNV particles were detected in all but one specimen, a cloacal swab taken from a Fish Crow. Viral titrations and quantitative TaqMan RT-PCR indicated that the concentrations of WNV averaged >10^5 in all three specimen types (Table).

Table. Mean logarithmic titers of West Nile virus (WNV) infectious particles, determined by Vero plaque assay and TaqMan reverse transcriptase–polymerase chain reaction[a]

Species	Specimen type (Mean Vero log PFU [range]/Mean TaqMan log PFU equivalents [range])		
	Brain	Oral swab	Cloacal swab
American Crow	8.2 [5.9–8.8]/ 7.1 [5.3–7.7]	7.3 [4.1–7.7]/ 6.6 [4.6–7.1]	6.4 [3.8–7.4]/ 6.9 [6.1–7.3]
Fish Crow	6.6 [4.1–6.9]/ 5.8 [4.8–6.2]	7.0 [1.4–7.6]/ 6.1 [3.2–6.7]	6.8 [<0.4–7.4]/ 6.0 [2.3–6.6]
Blue Jay	8.0 [7.3–8.2]/ 6.3[b] [6.2–6.3]	7.1[b] [5.3–7.4]/ 5.7[b] [4.4–6.0]	5.8 [3.0–6.3]/ 6.7[b] [5.6–7.0]

[a]In postmortem samples of brain tissue (1 cm^3), and oral and cloacal swabs for 12 American Crows, 4 Fish Crows, and 4 Blue Jays experimentally infected with the New York 1999 strain of WNV.
[b]This value determined from only two birds.

Conclusions

Avian mortality surveillance for WNV targets fresh carcasses (generally dead <24 h), especially corvids, for detection of infectious virus particles or RNA in brain or other viscera. We have shown that postmortem oral and cloacal swabs, in addition to brain, are effective samples to collect for WNV detection in experimentally infected corvids. A potential implication of these findings, pending field trials using corvids and other species routinely collected as part of avian mortality surveillance, is that WNV may be detected by simply collecting swabs from carcasses and forwarding the swabs (frozen) to a virology laboratory for testing. Eliminating multiple steps currently necessary for WNV testing of bird carcasses may conserve valuable public health resources and reduce the risk of exposure for laboratory personnel.

Acknowledgments

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*Centers for Disease Control and Prevention, Fort Collins, Colorado, USA; †Colorado State University, Fort Collins, Colorado, USA; and ‡United States Air Force, Bowling Air Force Base, Washington, DC, USA

[1]Pertaining to the family Corvidae, including crows, jays and magpies.

[2]These birds were infected either by mosquito bite or by direct contact with infected cagemates, both of which are potentially natural modes of infection.

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Nicholas Komar is the vertebrate ecologist for the Centers for Disease Control and Preventions' Arbovirus Diseases Branch, Division of Vector-Borne Infectious Diseases, in Fort Collins, Colorado. His major research interest is the role of vertebrate hosts in arbovirus transmission cycles.

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Address for correspondence: Nicholas Komar, Centers for Disease Control and Prevention, P.O. Box 2087, Fort Collins, CO 80522, USA; fax: 970-221-6476; e-mail: nck6@cdc.gov
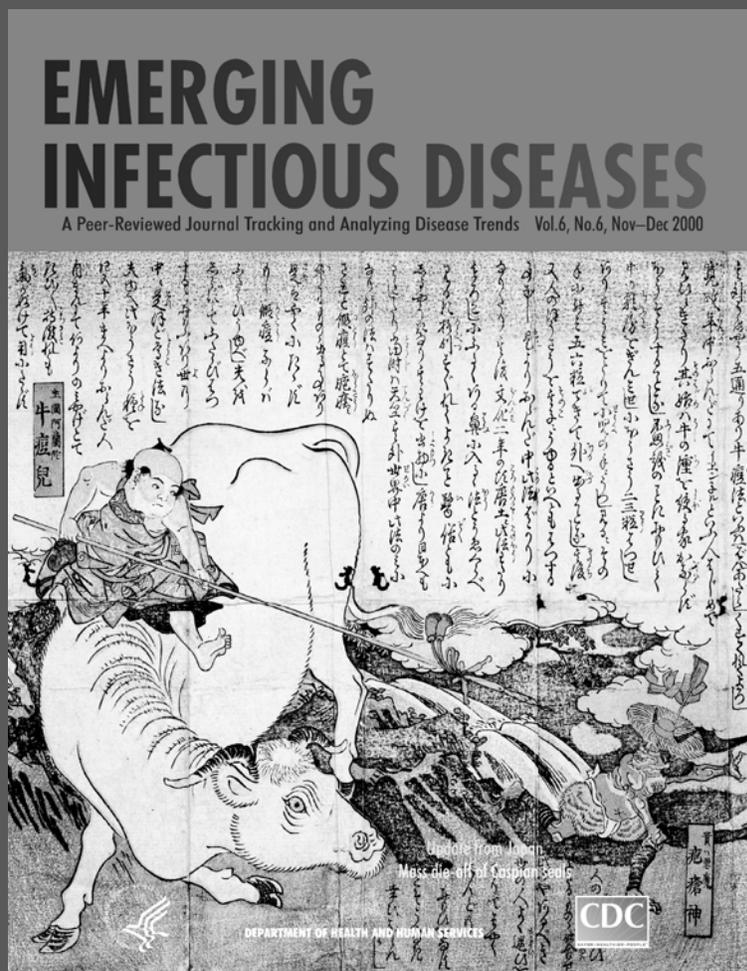
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Smallpox Research Activities: U.S. Interagency Collaboration, 2001

James W. LeDuc,* Inger Damon,* James M. Meegan,† David A. Relman,‡ John Huggins, § and Peter B. Jahrling§

For the past 2 years, a team of investigators from the Centers for Disease Control and Prevention (CDC) has collaborated with scientists from the National Institutes of Health (NIH), the Food and Drug Administration, the Department of Defense (DOD), academic centers, and international partners to undertake a research agenda on variola virus, the etiologic agent of smallpox. Objectives of the program derive from a 1999 Institute of Medicine report that addressed the scientific needs for live variola virus (1). Progress in addressing these objectives has been peer reviewed annually by both a select committee organized by CDC and the World Health Organization (WHO) Advisory Committee on Variola Virus Research (2,3). A summary of accomplishments from the first year's efforts was published in 2001 (4).

The events of September 11, 2001, coupled with the use of *Bacillus anthracis* as a bioterrorist weapon of mass destruction, have substantially increased concerns that variola virus may be similarly used and have added a sense of urgency to production of a new smallpox vaccine and to carrying out the smallpox research agenda. This report provides an update on progress during 2001.

Vaccine

CDC has worked closely with the manufacturer of Dryvax (Wyeth, Marietta, PA), the 20-year-old smallpox vaccine held in stockpile, to ready all 15.4 million doses of vaccine for immediate distribution. Stocks of this vaccine were retested for potency by the manufacturer and with very few exceptions were found to remain fully potent. However, problems were detected with the diluent used to rehydrate the freeze-dried vaccine, and consequently all diluent has been replaced. A recent NIH study found that Dryvax vaccine could be diluted 1:5 and 1:10 and remain fully potent when administered to vaccinia-naïve persons (5). Either a 1:5 or 1:10 dilution of vaccine can be made in the original vaccine container, without the necessity of transfer to larger vials. Emergency vaccination plans now call for the vaccine to be used at a 1:5 dilution; thus, the existing stockpile could protect approximately 75 million persons. Sufficient additional diluent and needles are being

purchased to support administration of the 75 million doses of vaccine.

To ensure that sufficient vaccine becomes available to protect the entire U.S. population, a contract established in 2000 by CDC with Acambis (Cambridge, MA) to produce and maintain a stockpile of 40 million doses of a new, MRC-5 (a diploid human lung cell line suitable for the production of viral vaccines) cell-culture–grown vaccinia vaccine was modified to reflect the need for expanded and accelerated production and human testing. The revised goal is now to produce >50 million doses by the end of 2002, with increased surge capacity for production of >180 million doses annually from 2003 on. Pilot lot production of the new vaccine is now under way, as are Phase 1 clinical trials. Phase 2 and 3 clinical trials are scheduled to begin later in 2002. Acambis expects to license the new vaccine by the end of 2003; however, it will be available for emergency use as an Investigational New Drug (IND) product as soon as it is manufactured. A second contract has been awarded to Acambis, in partnership with Baxter (Vienna, Austria), for production of 155 million doses of Vero cell-culture–produced vaccinia vaccine, for delivery by the end of 2002. The expanded use of the Dryvax vaccine, coupled with production of new vaccine from these two contracts, should result in sufficient vaccine for the entire U.S. population by the end of 2002.

Vaccinia vaccine is known to produce adverse events in a small number of recipients (6). In the spring of 2001, interested parties met to discuss management of vaccinia vaccine adverse events. Among the findings was the need to produce substantially larger stocks of vaccinia immune globulin, and suppliers are now being sought to produce approximately 30,000 adult treatment doses.

Antiviral Drugs

An important recommendation from the Institute of Medicine report (1) was to identify antiviral drugs with activity against variola and related orthopoxviruses. The research team has been working toward the goal of identifying two distinct antiviral drugs with different mechanisms of action and effectiveness in the treatment of smallpox infection. One candidate, cidofovir, has been identified and its activity against variola virus demonstrated in in vitro assays. During 2001, an IND was filed for use of cidofovir in both the treatment of acute smallpox infection and the management of adverse events

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †National Institutes of Health, Bethesda, Maryland, USA; ‡Stanford University, Stanford, California, USA; and §U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland, USA

associated with vaccinia immunization. The team examined selected analogs of cidofovir and found some to be 25- to 150-fold more active than the parent compound in in vitro assays. These promising candidates will be included in future assays with animal models. More than 700 additional candidate antiviral drug compounds have been screened by in vitro assays for activity to various orthopoxviruses. Of these, >20 were found to hold promise against vaccinia and variola viruses and will be tested in animal models. In addition, Eli Lilly and Company (Indianapolis, IN) has provided access to agents from their portfolio of compounds for screening against variola virus. Access to such compounds, which are already being used in humans and routinely produced, could dramatically accelerate efforts to identify antiviral compounds that would be useful against vaccinia and variola viruses.

Diagnostic Tests

The team continues to develop and validate assays to rapidly and accurately diagnose smallpox infection and the presence of variola virus, including protein-based tests to identify variola virus antigen and antivariola immunoglobulins, as well as specific nucleic acid detection tests. However, validation of prototype assays is hampered by the lack of access to relevant human clinical samples. DOD and international collaborating scientists worked successfully to identify a panel of monoclonal and polyclonal antibodies for detection and diagnosis of variola virus. An assay was developed for rapid and specific identification of variola virus with polymerase chain reaction (PCR) probes targeted to variola gene sequences. When these probes were used in the Smart Cycler (Cepheid, Sunnyvale, CA)/ TaqMan (Roche Molecular Systems, Inc., Indianapolis, IN) format, the limit of detection was validated by using the Bangladesh 1975 variola isolate and found to be approximately 483 copies. The assay was evaluated in a blinded study with 164 samples that included genomic DNA from 40 isolates of variola and 8 isolates of other orthopoxviruses at concentrations ranging from 0.1 to 10,000 pg/µL. No false-positive reactions were detected with any of the 37 nonvariola samples (100% specificity). Of the 127 samples containing variola DNA, 10 were considered negative, all at concentrations of ≤10 pg. The overall sensitivity was 92% at a limit of detection of 0.1 pg and 95% at a limit of detection of 1.0 pg. Preliminary data indicate that the assay is compatible with the ABI 7700 (Perkin-Elmer, Applied Biosystems Division, Foster City, CA) and the Light Cycler (Roche Diagnostic Corp., Indianapolis, IN) platforms, as well as colorimetric and electrochemical PCR enzyme-linked immunosorbent assay platforms. Collaborative work is continuing to evaluate and validate the assay to these various platforms.

Genetic Diversity of Variola Virus

The need for additional sequence analyses of variola virus isolates was recognized both by the Institute of Medicine expert review (1) and the WHO Advisory Committee on Variola Virus Research Expert Committee on Orthopoxviruses.

The WHO committee specifically recommended that, at a minimum, isolates from Congo and Somalia be fully sequenced before final destruction of variola virus. The team has developed a standardized method to extend PCR-restriction fragment-length polymorphism (RFLP) for the detection and differentiation of variola virus strains. From the WHO collection held at CDC, 45 variola isolates were selected on the basis of diverse geographic distribution and year of isolation. Twenty consensus primer pairs were used to produce 20 overlapping amplicons, which include 99.9% of the variola genome. The amplicons were digested with restriction endonuclease and resolved on acrylamide gradient gels, and the resulting RFLP patterns of the amplicons from the 45 isolates were compared. A composite dendrogram of all amplicon RFLP profiles differentiates variola major from variola minor, and the subclades within variola virus were generally clustered according to their geographic location or epidemiologic history. Unique RFLP patterns are generated on different amplicons of the variola isolates; these patterns can be used to differentiate variola strains and may be highly informative in epidemiologic studies. A database being built may also help in rapid recognition of variola or other orthopoxviruses that have been genetically engineered by the insertion of foreign genes.

On the basis of the WHO expert committee recommendations and the degree of diversity identified through the RFLP analyses, the team determined complete genomic sequences for eight isolates of variola virus from the WHO collection (named by geographic area and year of collection: Congo 70, Somalia 77, Nepal 73, India 64, Sumatra 70, Afghan 70, Horn 70, and Bangladesh 75). In addition to determining the full-length sequence of the most diverse isolates of variola virus, sequencing has been completed for >30 genes encoding various proteins from the other 37 isolates examined from the collection. All results and analyses are being integrated into a poxvirus-specific relational database suitable for public access (www.poxvirus.org). Finally, a new proteomics facility is being established at CDC to facilitate further characterization of variola virus isolates with the goal of providing essential information to assist in the rational design of antiviral drugs and therapeutic interventions. This facility is scheduled to be fully functional during 2002.

Animal Model for Smallpox

Clinical manifestations of human smallpox are well described (6). However, attempts to establish an animal model that faithfully replicates human smallpox have historically been unsuccessful. In 2002, the interagency team began evaluation of nonhuman primate models to facilitate in vivo evaluation of antiviral drugs and other therapeutic interventions. A valid animal model will generate important clinical material to assist in validating smallpox diagnostic tests and will allow detailed investigation of the pathogenesis of smallpox infection. Earlier efforts had succeeded in infecting cynomolgus macaques with variola virus but failed to produce clinical illness fully consistent with human smallpox. Exposures of

additional monkeys through a combination of both intravenous injection and aerosol exposure to high-titered (10^9 PFU) variola virus succeeded in inducing infection, which led to fatal disease after a clinical course like that of smallpox in humans. Subsequent infections by the intravenous route alone led to fatal infection at only the highest infecting dose (10^9 PFU), although monkeys receiving lower doses (10^6 to 10^9 PFU) did show evidence of variola infection, and virus dose was correlated with severity of disease course (7). Distribution of viral antigens by immunohistochemistry was correlated with replicating viral particles, observed by electron microscopy, and pathologic lesions resembling human smallpox. Analysis of daily specimens allowed detection of viral genomes in peripheral blood leukocytes and throat fluids by TaqMan PCR within 48 hours of exposure, suggesting the possibility of definitive diagnosis of smallpox during the prodrome. Overall, these results suggest that a valid animal model for smallpox may be feasible.

Collaborative studies with scientists from Stanford University allowed application of high-density DNA microarrays to measure and classify gene expression and to study the behavior of many genes simultaneously in monkeys experimentally infected with variola virus. Peripheral blood mononuclear cells were obtained from monkeys on the day of infection and multiple subsequent days. Gene expression analysis identified dramatic, highly choreographed response patterns and revealed several biological themes that appeared to be related to the outcome of infection (K. Rubins et al., Stanford University, unpub. data). Additional experimental infections of nonhuman primates with variola and other orthopoxviruses will be conducted. Potential benefits of host genome-wide expression profiling include early detection of infected persons, recognition of prognostic markers, rational development of novel therapeutic and prophylactic strategies, and determination of early signatures of a protective immune response to vaccination.

The team has continued investigating whether human chemokine receptors are involved as host factors in orthopoxvirus infections. Orthopoxviruses have no known unique viral attachment protein or cell surface receptor. Chemokine receptors, which are on the surface of leukocytes, are involved in directing leukocytes into areas of inflammation; other pathogens are known to use these receptors for cell entry. To determine the potential role of chemokines and their receptors in the pathogenesis of variola infection, experiments were performed by using a cell line transfected with genes encoding human CD4 receptors and subsequently transfected with human chemokine receptors. In low-multiplicity infection, the growth rate of variola was enhanced when human chemokine receptors were expressed. These and other observations suggest a possible role for chemokine receptors in the net growth and spread of variola virus. Investigations of the role of chemokines in the pathogenesis of variola infection will continue.

Conclusion

World Health Assembly resolution WHA52.10 called for the destruction of all remaining stocks of variola virus by the end of 2002; however, a recent WHO review of research progress concluded that the live virus would be required beyond that date, a position supported by the 55th World Health Assembly (8,9). Specific scientific priorities that remain to be addressed include 1) obtaining further sequence data from the terminal regions of additional variola isolates, 2) continuing efforts to effectively detect variola virus infection and validate these procedures, 3) developing new drugs for smallpox treatment, 4) developing less reactogenic vaccines to protect against smallpox infection, and 5) validating an animal model of human smallpox to allow assessment of candidate drugs and vaccines for both efficacy and regulatory purposes.

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High Risk for Tuberculosis in Hospital Physicians, Peru

To the Editor: Occupational exposure to *Mycobacterium tuberculosis* poses a major risk to medical staff worldwide. In areas of high tuberculosis (TB) incidence such as Peru (228–364 cases/100,000 [1,2]), the risk for hospital workers may be 40 times greater than that for the general population (3). Prospective studies to evaluate the precise occupational risk to medical staff in developing countries with a high incidence of TB disease are lacking. We evaluated the incidence of TB skin test (TST) conversion and TB disease in interns and residents in a teaching hospital in Lima, Peru.

Our study included 54 medical interns and 45 residents who began training in April 2000 at the Carrión Hospital, where all patients with TB are housed in wards without respiratory isolation. Each study participant had an initial evaluation before beginning hospital training. TST and chest radiographs were performed on entry into the study and 11 months later. TSTs were performed by the standard Mantoux technique, that is, intradermal injection of purified protein derivative (5 tuberculin units/0.1 mL) (Connaught Ltd., Ontario, Canada). Indurations were measured 48–72 hours later. A positive result was defined as an induration ≥10 mm. TST conversion from a negative to a positive result was defined as an increase of ≥10 mm in induration, according to criteria of the Centers for Disease Control and Prevention (CDC) (4). Every 3 months, physicians were screened for signs and symptoms of TB, and at 9 months they were interviewed for risk factors such as area of service, contact with active TB cases, and usage of an approved mask (N95 type). All statistical analyses were performed by using STATA 7.0 (Stata Corporation, College Station, TX).

Ninety-eight interns and residents (mean age 29.2 years ± 4.1 SD) were evaluated with an initial TST. One resident declined participation. Fifty-nine percent of the participants were TST positive at the initial evaluation. The presence of one BCG scar was not correlated with initially positive TST results. However, positivity in participants with two or more BCG scars was significantly higher (odds ratio 8.6; 95% confidence interval 1.8 to 79.5; p=0.002). Approximately two thirds (66/97) of participants recalled contact with an active TB case before the study period. All eight physicians whose relatives had been treated for TB were initially TST positive (p=0.01).

Of the 40 physicians who were negative at the initial TST, 35 (88%) were tested again 11 months later. Five residents did not have a TST. In one of these, an intensive-care resident, active pleural TB developed; the other residents remained well after 1 year. Five of the 35 physicians retested after 1 year had converted; 1 of these 5 also had pleural TB. Thus, 2 (5%) of 40 initially TST-negative physicians had acquired active TB, for an annual incidence of 2% (2/98).

The annual TST conversion rate for TST-negative physicians was 17% (6/36, including the TB patient without a follow-up TST). In addition, 11 (31%) of 35 persons with an initial negative TST result were positive (≥10 mm) on second testing, but the increase in induration was <10 mm. No significant differences were observed between conversion rates in interns and residents.

Ninety-five physicians responded to the questionnaire on mask use. Over 9 months, 87 (92%) of 95 trainees had treated patients with active TB (mean number of cases 14 ± 14 SD). In the TST-negative group who were retested, physicians who converted had significantly more contact with patients known to have active TB than physicians who did not convert (24 ± 11 SD, respectively, vs. 8 ± 7 SD, p=0.003). During this period of fol-low-up, no physician was aware of his having been exposed to a TB patient in the physician's home. No other risk factors (age, gender, area of service, participation in intubation or autopsy, approved mask usage, number of BCG scars) differed significantly between converters and nonconverters.

No physician reported consistently using a mask when examining patients with suspected TB or respiratory symptoms. Only 7 (7.4%) of 95 physicians reported that they consistently used a mask when examining active TB cases. Furthermore, 51 (54%) physicians never used a mask when examining a patient. Of the six physicians who converted, two reported never having used a mask, two reported mask use when working with diagnosed TB patients, and two reported sometimes using masks when working with TB patients.

Physicians exposed to a large number of TB cases at a public hospital had a 17% annual TST conversion rate. This rate is much higher than the 3% conversion rate in people living in a poor, overcrowded urban setting (2). Studies in industrialized countries show annual conversion rates ranging from 0.1% to 2% in unexposed employees and 1% to 10% in highly exposed health-care workers (5).

Our study also demonstrated a high incidence of symptomatic TB in Peruvian physicians. The 2% rate reported in this study is 10–15 times higher than that reported for the general population (6) and is similar to that in nurses caring for advanced TB patients in England during the 1930s (7).

The high TST conversion rate in physicians is most likely due to exposure to TB in the hospital. Boosting may at times produce large reactions and in all serial TB studies will be a potential confounder (8). To decrease the likelihood that boosting had occurred, we used stringent CDC criteria for conversion. The high incidence of active TB in the physicians strongly suggests that most conversions were due to TB transmission rather than boosting.

Additionally, the high conversion rate (two of five) in those who reported consistent mask use when caring for active TB cases may suggest overreporting of mask use, poor adjustment of the mask, contact with unsuspected active cases (9), or contact with a contaminated environment.

This high TST conversion rate and incidence of TB demonstrate the inadequacy of hospital infection control measures. In Peru, both unsuspected active TB and multidrug-resistant TB are highly prevalent (9). Rapid detection and respiratory isolation of patients with active or suspected TB are rarely practiced.

In conclusion, Peruvian physicians have an extremely high risk of TST conversion and active TB. Hospitals in developing countries need to design and implement effective and appropriate infection control measures such as appropriate mask usage, sputum testing, and rapid reporting of MTB smears of all patients with respiratory symptoms, as well as respiratory control for smear-positive TB cases (10).

Nilo Bonifacio,* Mayuko Saito,†‡ Robert H Gilman,†‡§ Fay Leung,† Nancy Cordova Chavez,* Jesús Chacaltana Huarcaya,* and Carlos Vera Quispe*

*Hospital Nacional Daniel A. Carrión, Lima, Peru; †Asociación Benéfica Proyectos en Informática, Salud, Medicina y Agricultura (Prisma), Lima, Peru; ‡Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD, USA; and §Universidad Peruana Cayetano Heredia, Lima, Peru

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First Documented Human *Rickettsia aeschlimannii* Infection

To the Editor: *Rickettsia aeschlimannii*, which was first isolated from *Hyalomma marginatum* ticks collected in Morocco in 1997 (1), has also been found in *H. marginatum* ticks from Zimbabwe, Niger, and Mali (2). For the past 3 years, we have included this species in the panel of rickettsiae for which sera from patients with suspected tickborne diseases are routinely tested. This procedure allowed us to document, by polymerase chain reaction (PCR) amplification and serologic testing, the first case of *R. aeschlimannii* human infection, which occurred in a patient returning from Morocco.

This 36-year-old man traveled to Morocco in August 2000. On returning to France, he noticed a vesicular lesion of the ankle, which became necrotic and resembled the typical "tâche noire" of Mediterranean spotted fever (3). He became ill with fever of 39.5°C and a generalized maculopapular skin rash. Laboratory tests showed a normal blood cell count but moderately increased transaminases. An early serum specimen was tested to confirm the diagnosis of Mediterranean spotted fever. By microimmunofluorescence, the patient's serum had immunoglobulin G and M titers of 1:32 and 1:16, respectively, against *R. aeschlimannii*; 0 and 1:16 against *R. conorii*, *R. africae*, *R. slovaca*, *R. helvetica*, and *R. massiliae*; and 0 and 1:8 against "*R. mongolotimonae*." Western blot results showed that the patient's serum reacted more intensively with *R. aeschlimannii* proteins than with those of the other tested rickettsiae. Attempted PCR amplification of a 630-nt portion of the rickettsial *ompA* gene (nt 70 to 701) (4) from the early serum specimen yielded a product of the expected size. The sequence of this amplicon allowed the identification of *R. aeschlimannii* with 100% homology. The patient was treated with doxycycline, 200 mg daily for 1 week, and rapidly recovered.

This case is the first documented infection caused by *R. aeschlimannii,* a *Rickettsia* that had been isolated only from *Hyalomma marginatum* ticks from Africa. In our patient, its pathogenic role was demonstrated by PCR, a technique that has also proven useful in identifying other new rickettsial diseases, including infections with

R. helvetica (5), *R. slovaca* (6), and *R. felis* (7). The serologic findings indicated antibodies at a higher level to *R. aeschlimannii* than to other tested species. *R. aeschlimannii* is phylogenetically distant from *R. conorii* but is closely related to *R. rhipicephali* and *R. montanensis*, which have never been described as human pathogens. This patient appeared to have a typical case of *R. conorii* infection, with seasonal and geographic characteristics favoring this diagnosis (3). This case was clinically and epidemiologically mistaken for *R. conorii* infection, suggesting that *R. aeschlimanii* may be another cause of Mediterranean spotted fever in Morocco.

The systematic identification of rickettsial species in human infections continues to increase the number of recognized human pathogens (3). This finding has demonstrated once again that more than one species or serotype of tick-transmitted rickettsia may be prevalent in the same area, as observed, for example, with *R. slovaca, "R. mongolotimonae,"* and *R. conorii* in southern France (3); *R. africae* and *R. conorii* in sub-Saharan Africa (8); and *R. conorii* and Israeli spotted fever rickettsia in Sicily and Portugal (9). *Rickettsia* species first identified in ticks should be considered as potential human pathogens, as all recently described tick-transmitted rickettsiae pathogenic for humans were initially found in ticks and were considered nonpathogenic for several years (3).

Didier Raoult,*
Pierre-Edouard Fournier,*
Philippe Abboud,†
and François Caron†

*Unité des Rickettsies, Université de la Méditerranée, Marseille, France; and †Service de Maladies Infectieuses, Centre Hospitalier Universitaire de Rouen, Rouen, France

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Cost-Effective Screening for Trichomoniasis

To the Editor: I read with interest a recent article in your journal, "*Trichomonas vaginalis*, HIV, and African Americans" (1), and I commend the authors' suggestion to implement screening and reporting of trichomoniasis for high-risk populations.

In the article, a cost-effective screening approach is mentioned, which includes culturing only for those women whose wet-mount tests are negative. In 1999, my colleagues and I reported on the validity of this method for diagnosing trichomoniasis in women (2). During our study, an additional vaginal swab was collected during the pelvic examination and placed into a glass tube. If the wet mount was negative, this swab was later added to a culture pouch for *T. vaginalis*. We found no statistically significant difference in the sensitivity of this method compared with that of adding swabs immediately to pouches at bedside. This method of delaying the second test until the results of the first test are known should be considered in screening women for trichomoniasis, especially in high-prevalence populations.

Jane R. Schwebke

University of Alabama at Birmingham, Birmingham, Alabama, USA

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Reply to Dr. Schwebke

To the Editor: We welcome Dr. Schwebke's thoughtful comments about decreasing the cost of screening for *Trichomonas vaginalis*. Dr. Schwebke and her colleagues have demonstrated that storing a vaginal swab for 15–20 minutes in a glass tube at room temperature does not affect the viability of *T. vaginalis* or reduce the sensitivity of subsequent culture. This finding shows that vaginal swabs may be stored briefly while a wet-mount preparation is made and examined. If the wet mount is negative for *T. vaginalis,* the stored swab can then be processed for culture. If the wet mount is positive for *T. vaginalis,* no further culture of the specimen is needed, thereby reducing unnecessary costs. Given that the prevalence of this infection often exceeds 20% in high-risk populations, this approach can reduce costs substantially without compromising the accuracy of the tests. Any method that reduces the cost of diagnosis will advance further

screening for trichomoniasis and promote the ultimate goal of implementing intervention efforts.

Frank Sorvillo,*† Lisa Smith,*† and Peter Kerndt†

*University of California at Los Angeles, Los Angeles, California, USA; and †Department of Health Services, Los Angeles County, Los Angeles, California, USA

Hot-Tub–Associated Mycobacterial Infections in Immunosuppressed Persons

To the Editor: I read with interest the report by Mangione et al. regarding *Mycobacterium avium* infection in a Colorado family who used an inadequately sanitized hot tub (1). The authors noted that the source of the *M. avium* complex was not clear, although the reservoir did appear to have been the hot tub.

Twenty years ago, I helped treat a patient with a local infection caused by *M. fortuitum* in his amputation stump (2). The patient had sat in his tub postoperatively three to four times per week. Although he had added disinfectants as recommended by the manufacturer, he had not cleaned the tub mechanically at any time during the incubation period of his infection. We recovered what appeared to be the same strain of *M. fortuitum* from the abscess on his amputation stump and specimens from the hot tub water and filter. However, we could not recover any mycobacteria from his or his neighbor's tap water.

Three years after our experience with this patient, *M. chelonei* was found to cause colonization of sputum of patients with cystic fibrosis after they had been treated in a hydrotherapy pool (3).

Very recently, an outbreak of 110 cases of furunculosis was attributed to *M. fortuitum* contamination of a footbath at a nail salon (4).

These experiences indicate the absolute need for careful cleaning of hot tubs. Not only are immunosuppressed patients at risk for atypical mycobacterial infections but even otherwise healthy persons may be susceptible.

Donald R. Graham

Springfield Clinic, Springfield, Illinois, USA

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EMERGING www.cdc.gov/eid
INFECTIOUS DISEASES
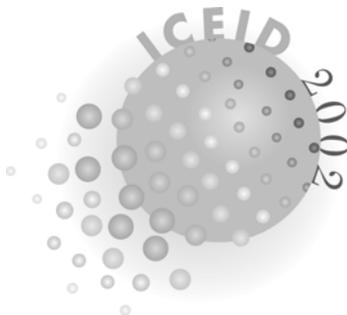
JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

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

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

Why an "Emerging" Infectious Diseases journal?

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

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

What are the goals of Emerging Infectious Diseases?

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

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

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

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

About the Cover

Tametomo no bui toukigami wo shirizoku zu

Ukiyo-e woodcut print by Yoshitoshi, c. 1890

**Naito Museum of Pharmaceutical
Science and Industry**

Hashima, Gifu, Japan

The first record of smallpox in Japan was found in the Nihon Shoki, published in 735 (the 7th year of Tempyo). The incident was also described in Ishinho, the oldest medical book in Japan, issued by Yasuyori Tanba in 984 (the 2nd year of Eikan). Smallpox, called Hoso in Japanese, came to Japan in the same era as Buddhism. The disease was considered very dangerous. Even those who recovered could have pockmarks or loss of sight. Parents were constantly concerned about their children becoming ill with smallpox.

The color red was used in prints and other smallpox illustrations because it was believed that Hoso-Kami, the god of smallpox, felt strongly about this color. When the skin rash was purple, the patient's condition was considered serious. If the rash turned red, the patient would recover safely. Shoni-Hitsuyo-Yoikugusa, written by Gyuzan Kazuki in 1798 (the 10th year of Kansei), recommended that children with smallpox be clothed in red garments and that those caring for the sick also wear red.

"Hoso-e" color prints against smallpox were used in prayers to boost the morale of ill children. After the patients recovered, these pictures were burned or floated down the river. Therefore, few examples are left of prints in which the color red predominates. The pictures drawn as protection against smallpox depicted heroic figures to give people courage against smallpox. Tametomo, a heroic samurai, was a representative genie. Legend has it that Tametomo was once banished to Hachijyo Jima, a small island far from main island in Japan, and that is why smallpox never occurred there.

Reference

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In the next issue

Passive Antibody Administration (Immediate Immunity) as a Specific Defense Against Biological Weapons

West-Nile Outbreak in Horses in Southern France, 2000: Results of a Serosurvey

Outbreak of Cyclosporiasis Associated with Imported Raspberries, Philadelphia, Pennsylvania, 2000

Genetic Homogeneity of Measles Viruses Associated with a Measles Outbreak, São Paulo, Brazil

Genetic Characterization of Hantaviruses Carried by the Korean Field Mouse (*Apodemus peninsulae*), Far East Russia

Haemophilus Aphrophilus Endocarditis Following Tongue Piercing

Infantile Pertussis Rediscovered in China

Jet Black Eschar

Polymerase Chain Reaction for Screening Blood Donors at Risk for Malaria: Safe and Useful?

A New Pathogenic Spotted Fever Group Rickettsia in South Africa

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

Editorial Policy and Call for Articles

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

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

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

Chinese, French, and Spanish translations of some articles can be accessed through the journal's home page at http://www.cdc.gov/eid.

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and e-mail address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Printed manuscript should be single-sided, beginning with the title page. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word's table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Submit an electronic copy (by e-mail) to the Editor, eideditor@cdc.gov.

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two. Report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be 1,000–1,500 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed two); and a brief biographical sketch of first author—both authors if only two. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentary. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but should not include figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

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

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

News and Notes. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.) In this section, we also include summaries (500–1,000 words) of emerging infectious disease conferences. Summaries may provide references to a full report of conference activities and should focus on the meeting's content.