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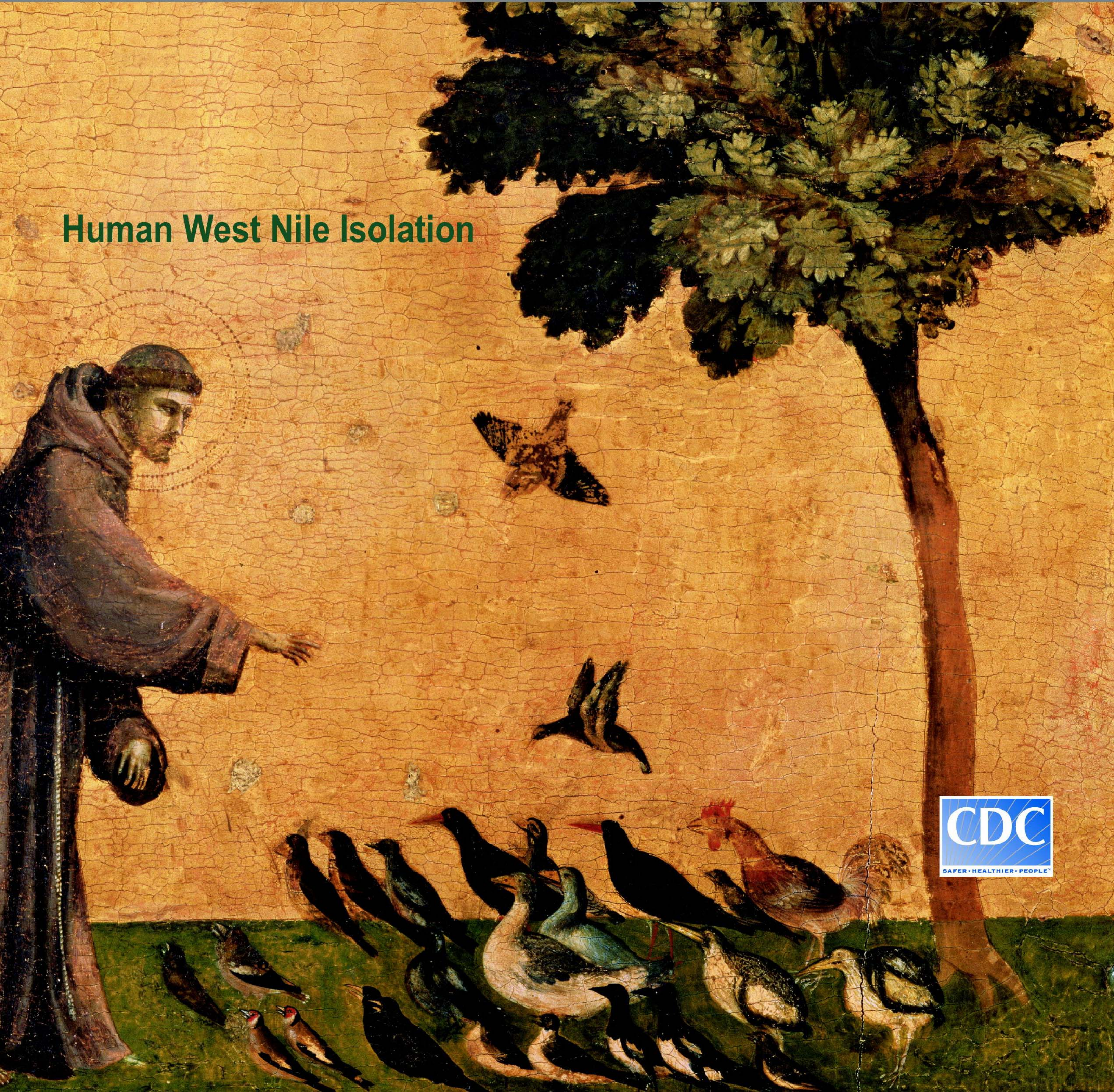

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Giotto di Bondone (c. 1267-1337).
St. Francis of Assisi
Receiving the Stigmata (c. 1290).
Tempera on wood, 313 cm x 163 cm.
Musée du Louvre, Paris, France

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First Isolation of *West Nile virus* from a Patient with Encephalitis in the United States

Cinnia Huang,* Brett Slater,* Robert Rudd,* Nandakishore Parchuri,† Rene Hull,* Michelle Dupuis,* and Alexander Hindenburg†

West Nile virus (WNV) was isolated from a patient who developed encephalitis while undergoing treatment with CHOP (cyclophosphamide, hydroxydoxorubicin, vincristine [Oncovin], predisone) and rituximab for a non-Hodgkin B-cell lymphoma. Both standard reverse transcription–polymerase chain reaction (RT-PCR) and Taqman RT-PCR established the diagnosis of WNV infection from cerebrospinal fluid (CSF). Several whole blood samples and one serum sample underwent further testing. CSF and serum samples were negative for WNV antibody; however, all samples were positive by both RT-PCR assays. Infectious virus was recovered from a blood sample, and its identity was confirmed by using a WNV-specific immunofluorescence assay. The complete WNV genomes determined from CSF and from the virus isolate adapted from cell culture were the same. The results represent the first complete WNV genome sequence obtained directly from human CSF and the first time that infectious WNV has been recovered from a patient with encephalitis in North America.

West Nile virus (WNV), an arthropod-borne virus, is a member of the Japanese encephalitis virus serocomplex of the genus *Flavivirus*, family *Flaviviridae* (1), discovered in Uganda in 1937 (2). Although WNV infections are usually mild or asymptomatic, in some instances, a severe and fatal encephalitis is produced, typically in the elderly (3). This virus was first recognized in the Western Hemisphere in an outbreak in New York in 1999 (4). As of October 2, 2002, a total of 2.671 cases of human illness in the United States have been reported to the Centers for Disease Control and Prevention (CDC). Although WNV has been recovered from mosquitoes, birds, and horses, no isolations from humans have been reported in the Western Hemisphere. Thus, all prior data regarding the virus responsible for human illness in North America have come from nucleic acid sequencing of the viral genome in either cerebrospinal fluid (CSF) or brain tissue. We describe the first isolation of WNV from a human case-patient associated with the U.S. outbreak. Because the virus was also directly detected by reverse transcription–polymerase chain reaction (RT-PCR) in specimens from the same patient, we were able to compare the entire genomic sequence of the directly detected virus with that of the cell-culture isolate.

Case Report

The patient was a 70-year-old woman, who has been diagnosed with intermediate-grade, CD 20-positive, B-cell non-Hodgkin lymphoma, involving a left intraparotid lymph node. Staging workup demonstrated stage 1-A disease. Treatment plans for the patient included three courses of CHOP (cyclophosphamide, hydroxydoxorubicin, vincristine [Oncovin], and predisone) chemotherapy plus rituximab (chimeric CD 20 monoclonal antibody), to be followed by involved field radiation. After the first cycle of chemotherapy in July 2001, neutropenia developed and the patient was treated with granulocyte colony stimulating factor (GCSF) following both the second and third cycles of chemotherapy. The third cycle of chemotherapy was administered on September 11, 2001. Four days later, she was treated with oral levofloxacin for low-grade fever. On day 7 after chemotherapy, she was admitted to the hospital with fever, cough, chills, rhinorrhea, joint aches, decreased appetite, lethargy, and lightheadedness. She had no recollection of any ill contacts or insect bites. She was a resident of southern Nassau County, New York, and did not have a history of travel. Physical examination was within normal limits. The patient was neutropenic; G-CSF was continued, and she was given cefipime and gentamicin. One day after admission, she continued to have fever and experienced mild headaches that responded to acetaminophen. Urine cultures showed penicillin-sensitive enterococci, and blood cultures were negative. Two days after admission, the patient continued to have fever, headaches, and dizziness. A computed tomography (CT) scan of the brain revealed no acute cerebral processes. On the 3rd day after admission, the patient was noted to be confused. Further deterioration of mental status was noted, with incomprehensible speech, but she was able to follow commands. Arterial blood-gas analysis showed an acute respiratory acidosis pattern, and the patient was subsequently intubated and transferred to the intensive care unit. The patient had a hypotensive episode secondary to atrial flutter, which required cardioversion for stabilization. Ceftriaxone and ampicillin were added to the antibiotic coverage, and antifungal treatment was also initiated with lipid complex amphotericin B. After

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the patient's hematologic parameters improved, G-CSF was discontinued on day 9 of hospitalization, and the patient continued to be afebrile. Because of the clinical picture of encephalitis and because the patient lived in an area where WNV was endemic, lumbar puncture was performed on day 8, and a CSF specimen was sent to the New York State Department of Health laboratories for comprehensive PCR testing. Renal tubular necrosis developed in the patient, leading to acute renal failure by day 10, with further deterioration of her mental status. Dialysis was initiated on day 16, and antibiotic therapy was discontinued on day 17 as the patient remained afebrile and the neutropenia resolved. The patient remained unresponsive, staphylococcal septicemia developed, and she died on day 35 of hospitalization. Autopsy showed a small focus of perivascular lymphocyte cuffing in the mamillary bodies of the brain, consistent with viral encephalitis. No evidence of residual lymphoma was found.

Materials and Methods

The PCR Laboratory associated with the Virology Diagnostic Services, Wadsworth Center, New York State Department of Health, has developed a panel of PCR and RT-PCR assays that allow tests on CSF and brain tissue for a wide range of viruses associated with human central nervous system (CNS) infections. Specifically, the test battery includes herpes simplex viruses (types 1 and 2), varicella-zoster virus, cytomegalovirus, Epstein-Barr virus (*Human herpesvirus 4*), enteroviruses, and the following arboviruses: Eastern equine encephalitis, California serogroup (LaCrosse virus [LACV], Jamestown Canyon, and others), *Powassan virus* (POWV), *St. Louis encephalitis virus* (SLEV), WNV, and Cache Valley virus.

RNA and DNA were simultaneously extracted from 0.25 mL of CSF sample with Trizol LS reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, RNA was first transcribed into cDNA with random primers (Roche Diagnostics Corp., Indianapolis, IN), and an aliquot of this cDNA (5 µL) was used in PCR reactions with primers for detecting viruses in the test panel. Aliquots of DNA were also examined for the presence of the herpesviruses in the panel. Amplification products were analyzed on a 2% agarose (EM Science, Gibbstown, NJ) gel containing ethidium bromide. Routinely, for any virus for which a band corresponding to a positive result is observed, the band is run into low melting agarose, and sequenced directly on the gel slice without further purification. DyeTerminator sequencing was performed on an Applied Biosystems Model 373A automated sequencer (Foster City, CA). For the TaqMan assay, one-step RT-PCR Ready-Mix Kit (Applied Biosystems) was used. The primers and probe for the quantification of the RNA copy number of WNV used in this study are listed in Table 1.

Virus was isolated by using monolayers of Vero cells grown in tubes. Aliquots of 0.01 and 0.05 mL of whole blood were pretreated with antibiotics (penicillin and streptomycin) for 30 min at room temperature (22°C); 1 mL of Eagle's minimal essential medium containing 2% fetal bovine serum was added to the treated blood samples, and the mixtures were used to inoculate the Vero cell monolayers.

After incubation for 24 hr at 37°C, the monolayers were rinsed with phosphate-buffered saline, 2 mL of fresh medium was added, and the culture was subsequently monitored daily for cytopathic effects (CPE). Cell monolayers that showed CPE were harvested. To confirm that the infectious agent was WNV, an aliquot of the supernatant serially diluted (10^{-4}–10^{-6}) was used to infect fresh Vero cell monolayers. Virus-infected monolayers from the second passage were examined for WNV antigen by immunofluorescence assay (IFA) by using monoclonal antibody H5-46 (provided by CDC, Fort Collins, CO). This monoclonal immunoglobulin (Ig) M antibody is specific for a glycoprotein epitope on WNV.

Results

Diagnosis of WNV Infection

CSF was simultaneously examined for a panel of 11 viruses by RT-PCR/PCR as described in Material and Methods. No amplification product was observed for any other virus in the PCR battery except WNV. Two primer pairs (CU9093/CL9279 and D87F/D156R) in the NS_5 region (5,6) of the WNV genome were used in the initial screening by standard RT-PCR (Table 1). PCR products of appropriate size for both primer pairs were obtained (Figure 1). RT-PCR was independently repeated on another aliquot of CSF by using four primer pairs located in var-

Table 1. Oligonucleotide primers and probe used in the standard RT-PCR and TaqMan assays[a]

Primer	Genome target	Genome position[b]	Sequence (5′–3′)	RT-PCR product size (bp)
CU9093	NS_5	9097–9120	AGYMGRGCHATHTGGTWYATGTGG	206
CL9279	NS_5	9302–9283	TTCCAVCCDGCKGTRTCATC	
D87F	NS_5	10034–10051	GCTCCGCTGTCCCTGTGA	70
D156R	NS_5	10103–10083	CACTCTCCTCCTGCATGGATG	
Forward	ENV	1160–1180	TCAGCGATCTCTCCACCAAAG	70
Reverse	ENV	1229–1209	GGGTCAGCACGTTTGTCATTG	
Probe	ENV	1186–1207	TGCCCGACCATGGGAGAAGCTC	

[a]RT-PCR, reverse transcription–polymerase chain reaction.
[b]Genome position according to GenBank accession no. AF196835.

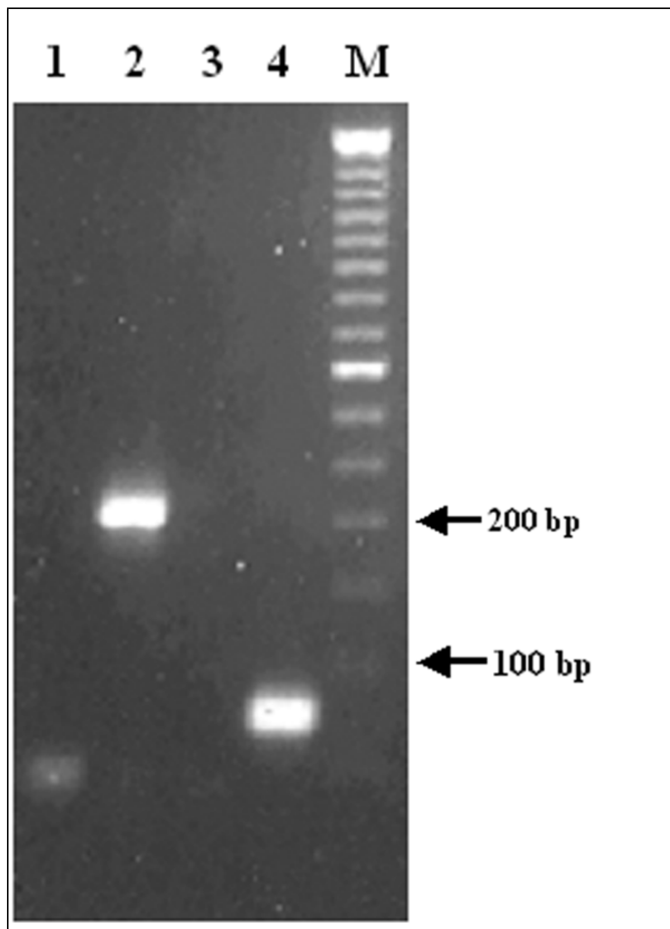

Figure 1. RT-PCR detection of *West Nile virus* RNA in cerebrospinal fluid. Lanes 1 and 3: negative controls; lanes 2 and 4: cerebrospinal fluid; lane M: 50-bp DNA ladder. Primer pairs used: lanes 1 and 2: CU9093/CL9279; lanes 3 and 4: D87F/D156R.

ious genomic regions of WNV. PCR bands with the expected sizes were present in all four reactions (data not shown). The identity of the PCR bands was confirmed by sequence data obtained directly from the PCR amplicon. The diagnosis of WNV infection was based on the detection and sequence of the WNV genome in CSF. Although the serologic test (IgM capture enzyme-linked immunosorbent assay [ELISA]) on the CSF sample was negative for WNV, according to the interpretations set forth by CDC, the detection of viral genome sequence in CSF meets the definition of a confirmed case.

TaqMan Assays

To follow up this case, three whole-blood samples and one serum sample were examined by both standard RT-PCR (primers in NS_5 region) and TaqMan (primers in ENV region) assays; the results are summarized in Table 2. The highest RNA copy number, 2.5×10^6 copies/mL, was found in the blood sample that was collected 3 days after the patient's neurologic symptoms appeared. WNV genome was also detected in a serum sample collected on day 19 after onset of symptoms. Serologic tests (IgM capture ELISA and IgG ELISA) on serum for *Eastern equine encephalitis virus*, LACV, POWV, SLEV, and WNV were all negative.

Other Clinical Data

Laboratory studies of the CSF indicated the following values: leukocyte (WBC) count 8 mm^3 with 66% neutrophils, 4% lymphocytes, 4% atypical lymphocytes, and 26% monocytes; erythrocyte count 0; glucose level 76 mg/dL; total protein level 55 mg/dL, and lactate dehydrogenase level 35 IU/L. Figure 2 presents fever curve, WBC curve, and viremia data. The serum immunoglobulins at the time of infection with WNV were IgG 492 mg/dL (normal level [nl]) 700–1,500 mg/dL), IgA 86 mg/dL (nl 65–450 mg/dL), and IgM 80 mg/dL (nl 45–230 mg/dL).

Virus Isolation

The attempt to isolate WNV was carried out in a biosafety level 3 laboratory not routinely used for arbovirus work and located in a building separate from the facility where PCR testing was conducted. This procedure had the dual advantage of minimizing the possibility that any virus recovered originated from a source other than the human specimen and also ensuring that any isolate obtained would not lead to future spurious PCR results. A blood sample collected on September 24, 2001, and a CSF specimen collected on September 26, 2001, were chosen for recovery of the virus because of the presence of high-copy-number viral RNA. On day 6 postinfection, CPE was observed in the tubes inoculated with 0.01 mL and 0.05 mL of blood, but not in the tube inoculated with 0.1 mL of CSF. WNV was confirmed in the second-passage cell cultures by IFA by using WNV-specific monoclonal antibody H5-46.

Table 2. Detection of *West Nile virus* in human specimens by TaqMan and standard RT-PCR assays[a]

Specimen	Collection date	RNA (copy/mL)	C_T	Rn	STD RT-PCR	Serology	Cell cultures
Blood	9/21/2001	2.2×10^3	31.6	0.48	Positive	n.d.	n.d.
Blood	9/24/2001	2.5×10^6	20.4	3.71	Positive	n.d.	Positive
CSF	9/26/2001	1.1×10^6	20.4	3.71	Positive	Negative	Negative
Blood	10/02/2001	5.4×10^4	25.8	2.70	Positive	n.d.	n.d.
Serum	10/10/2001	3.7×10^3	28.5	0.70	Positive	Negative	n.d.

[a]C_T, threshold cycle number, the cycle number at which fluorescence increases above a fixed threshold value; Rn, normalized fluorescent signal, the fluorescent signal generated by the reporter dye; STD, standard; RT-PCR, reverse transcription–polymerase chain reaction; n.d.: not done, CSF, cerebrospinal fluid.

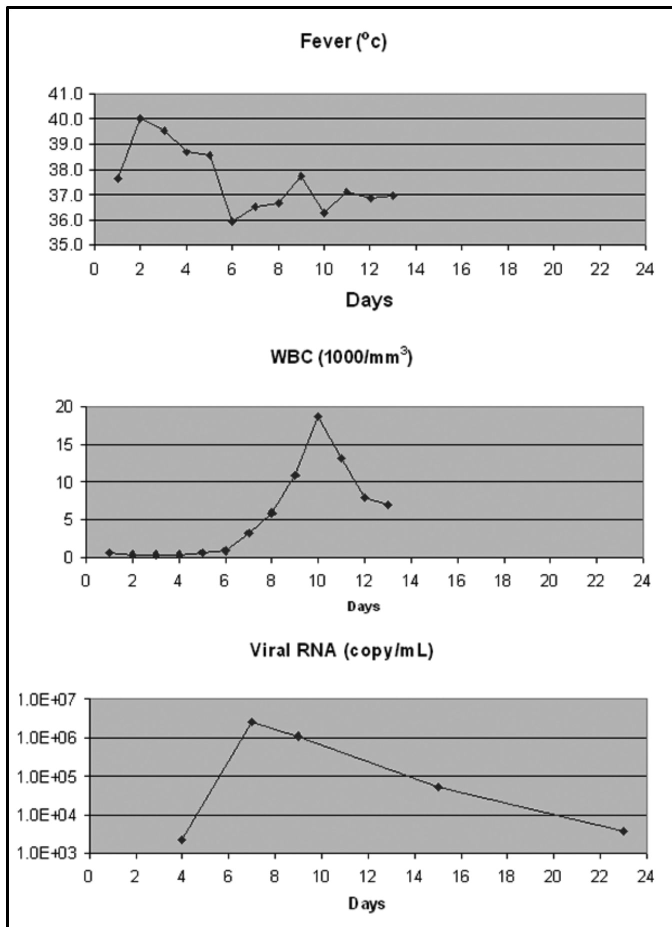

Figure 2. *West Nile virus* copy numbers in clinical samples and clinical. WBC, leukocytes. Detailed sample information is listed in Table 2; day 1 is date the patient was hospitalized, 9/18/2001.

Sequence Analysis

Since only a very small amount of CSF remained after the diagnostic work-up, we carefully designed a protocol to generate PCR bands with sizes ranging from 500 to 1000 bp. This protocol allowed the complete genome sequence of the virus in the CSF to be determined by sequencing overlapping PCR bands (GenBank accession no. AF533540). We used a similar protocol to obtain the genome sequence of the isolate adapted from cell culture and found that the sequence data from the virus in the CSF and from the WNV isolate were identical. A 1648-bp fragment encoding the PreM, M, and part of the 5′-E gene was used for phylogenetic studies (Figure 3). The analysis showed that the sequence data from this case are similar to the sequence data obtained from human (7) (GenBank accession no. AF202541), horse (8) GenBank accession no. AF260967), and bird (9) (GenBank accession no. AF196835) WNV isolates in New York in 1999.

Discussion

This case is important for several reasons. It represents the first instance in which WNV was recovered from a person in the United States. It is also the first time that the entire genomic sequence of WNV has been obtained from CSF from a human case-patient. The sequence data from the virus directly detected in the CSF and from the WNV isolate from cell cultures are identical. Hindiyeh et al. (10) reported the isolation of WNV from the blood of viremic patients who were not immunocompromised and who seroconverted later. In contrast, the patient in this case was elderly and was undergoing treatment for lymphoma. She was unable to mount an immune response as shown by the fact that the results of serologic tests on both CSF and serum specimens were negative.

To our knowledge, all previous attempts to recover WNV from human patients associated with the North American outbreak have been unsuccessful. The ability to recover an infectious isolate in this report may have been contingent on the fact that the patient was immunologically impaired. She had lymphoma and had been undergoing treatment with CHOP plus rituximab for 2 months at the time the WNV infection

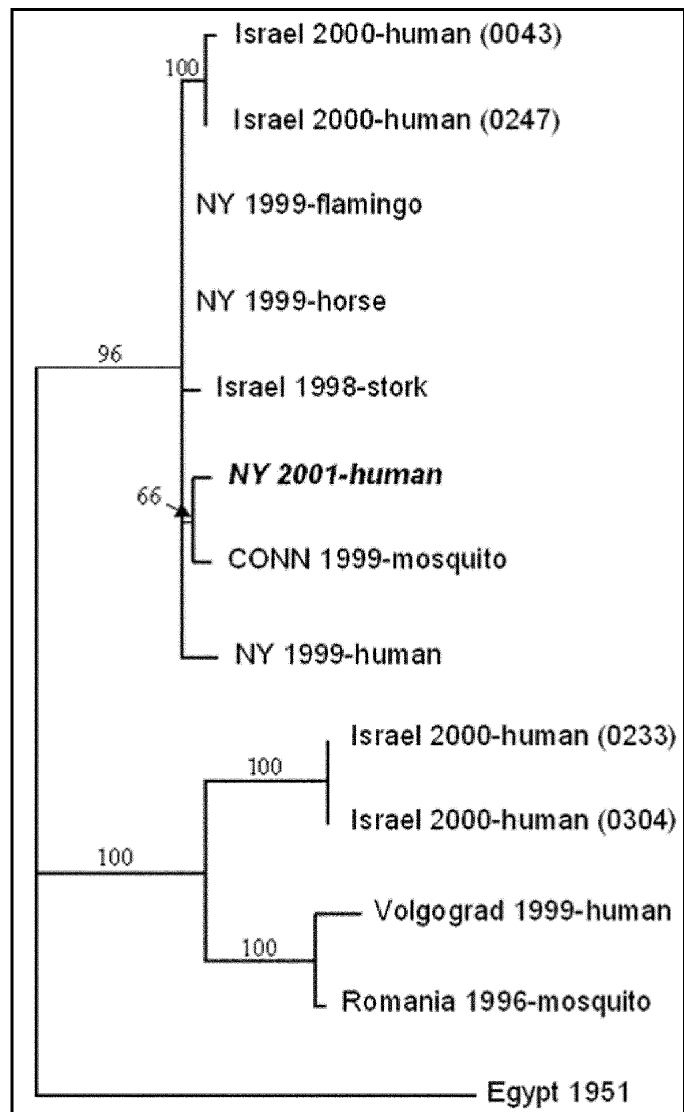

Figure 3. Phylogenetic relationships among *West Nile virus* strains. Sequence data from the present case are shown in italics. The tree is based on the 1,648-bp fragment encoding the preM, M, and part of the 5′-E gene. Numbers at the nodes are bootstrap confidence estimates based on 1,000 replicates.

developed. The relative role of immunosuppression caused by the lymphoma itself compared with the immunosuppression due to treatment of the lymphoma is unclear. At the time of the WNV infection, the patient's serum IgG levels were moderately suppressed. This was most likely the result of lymphoma, because a reduction in immunoglobulins, secondary to impaired B-cell function from rituximab, usually occurs after 3 months of therapy (11). In a previous randomized study, elderly patients receiving CHOP chemotherapy and rituximab had increased their overall survival and had not experienced an increase in toxic clinical effects compared with effects from CHOP treatment alone (12). However, rituximab used as a single agent has been reported to lead to excessive bacterial and viral infections, including respiratory tract infections and herpes (13). Rituximab is also implicated as a risk factor for unusual viral infections when used as an immunotherapy agent in the peritransplant period of autologous stem cell transplant in non-Hodgkin lymphoma patients (14). Another consideration is the fact that the patient was neutropenic. The relationship between neutrophil function and the severity of WNV infection is unknown. The virus may be cleared by neutrophils, and the severity of the viral infection may have been due to the fact that the patient was neutropenic at the time of the acute infection. What lends credence to this hypothesis is the observation that the highest viral titer as determined by PCR coincided with the recovery of the WBC count. Following the resolution of the myelosuppression, the RNA copy number of the WNV in blood samples declined rapidly (from 1.1×10^6 to 5.4×10^4 copies/mL).

In summary, this report is the first of WN encephalopathy in an immunocompromised patient undergoing treatment for lymphoma. The patient's serologic tests remained negative, and the diagnosis was made by RT-PCR from both CSF and peripheral blood, and by in vitro cultivation of the virus from blood. WNV infection should therefore be considered in the differential diagnosis of patients with lymphoma who exhibit encephalopathy, even if serologic tests are negative for WNV. Extra care should be taken to prevent patients with lymphoma, especially those undergoing treatment, from being exposed to WNV.

Acknowledgments

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RESEARCH

West Nile virus Epidemic in Horses, Tuscany Region, Italy

Gian Luca Autorino,* Antonio Battisti,* Vincent Deubel,† Giancarlo Ferrari,* Riccardo Forletta,* Armando Giovannini,‡ Rossella Lelli,‡ Severine Murri,† and Maria Teresa Scicluna*

During the late summer of 1998, veterinary authorities in Tuscany, Italy, received reports of cases of neurologic disease among horses residing in a large wetland area located in the provinces of Florence and Pistoia. *West Nile virus* was isolated from two of the six horses that died or were euthanized. A retrospective epidemiologic study identified 14 clinical neurologic cases that occurred from August 20 to October 6 (attack rate of 2.8%). A serologic survey conducted over a 700-km^2 area in stables with and without apparent clinical cases confirmed a wider spread of the infection, with an overall seroprevalence rate of 38% in the affected area. No significant differences in age-specific prevalence were observed, suggesting that the horses residing in the area had not been exposed previously to *West Nile virus* and supporting the hypothesis of its introduction in the wetland area during the first half of 1998.

West Nile virus (WNV), named after the district of Uganda where the virus was first isolated in 1937 (1), is a mosquito-borne *Flavivirus* belonging to the Japanese encephalitis antigenic complex in the family *Flaviviridae* (2). WNV has been described in Africa, Europe, Middle East, Asia, Oceania (subtype Kunjin), and, more recently, in North America (3).

The ecologic aspects of WNV infection, involving mosquitoes, birds, and humans, were first described in the 1950s in Egypt (4). The agent circulates in nature through continuous enzootic transmission cycles between *Culicinae* mosquitoes and avian vertebrate hosts and may be introduced into a new territory by migratory birds. Humans and horses are considered incidental hosts. However, an urban cycle with virus amplification by continuous transmission between birds and vectors, and incidentally, humans, has been recently described in Romania and in the United States (5–7).

The infection in humans is usually asymptomatic; however, in 20% of cases, an acute, influenza-like, self-limiting febrile illness may occur, with symptoms of severe encephalitis in <1% of the cases (8). Neurologic disease in both humans and horses was reported for the first time in the late 1950s (9) and 1960s (10,11) respectively, in the Mediterranean area. In the past decade, outbreaks in humans have been reported in Algeria in 1994 (12), in Romania in 1996 and in 1997–1998 (4,13), in the Czech Republic in 1997 (14), in Tunisia in 1997 (H. Triki, pers. comm.), in Russia in 1999 (15), and in birds, humans, and horses in Israel in 2000 (16). In the summer of 1999, the first recorded appearance of WNV in the Western Hemisphere caused fatal disease in humans, horses, and birds in the northeastern United States (5,6,17–19). Outbreaks in horses were described in Morocco from August to mid-October 1996 (20) with 94 cases and 42 deaths, and in France from August to November 2000 (21) with 58 confirmed cases and 20 deaths.

During the late summer in 1998, veterinary practitioners in Italy recorded an increasing number of cases of neurologic disease in horses around an 18,000-km^2 wetland area located in the provinces of Florence and Pistoia, known as the Padule del Fucecchio. The area, which covers the central and southern part of the Valdinievole Valley, is home to a number of resident and migratory avian species and is on the route between African wintering areas and European breeding sites of waterfowl, herons, and waders, some of which breed there during the summer.

On the basis of preliminary investigation by public health veterinarians, an outbreak of WNV disease in horses was suspected. On October 19, 1998, the Regional Veterinary Authority banned the movement of *Equidae* in and out of a 700-km^2 area including 20 municipalities. Preliminary epidemiologic and laboratory studies implicated WNV as the etiologic agent, and the ban was lifted 1 month later on November 20, 1998. By this point, the temperature in the area had dropped below levels at which *Culicinae* mosquito multiplication can occur, and no further cases had been reported since early October. The objectives of our study were to assess whether an epizootic was occurring among horses residing in the area and to perform a cross-sectional serosurvey to gather information about the extension of virus circulation around the wetland area, once as the cause of the infection was ascertained.

Methods

Retrospective Study

At the end of September 1998, following the initial occurrence of neurologic cases of unknown origin in horses, we initiated a retrospective study to assess possible common

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exposures. A questionnaire was prepared and distributed to public health veterinarians and veterinary practitioners, who were asked to complete it if they had seen or treated horses during the summer that matched the following case definition: "clinical signs involving the central or peripheral nervous system." This definition, although broad in scope, was intentionally used to include all possible cases in the course of the investigation. We collected information about location and size of the stable in which the case had occurred; the number, sex, age, and breed of horses present; a checklist of neurologic signs; and the date of onset, duration, and outcome of the clinical course. Additional information requested included whether the horse had received medical treatment within the 15 days preceding the clinical onset and whether any horse had been moved into or out of the stable within 30 days of the date of onset of clinical signs.

During the retrospective study, blood samples were collected in October from 159 horses in the stables where clinical cases had occurred. At an interval of 30 days, a second round of sampling was performed on 124 horses from the same stables. Repeat titers were obtained, from 123 (78%) of the 159 animals bled in October. Further blood samples (n=161) were collected in 16 stables in proximity with those with clinical cases. A confirmed case was defined as a horse with neurologic signs suspect of WNV origin, together with at least one of the following: isolation of WNV, a positive polymerase chain reaction (PCR) assay, and a positive complement fixation (CF) test. A complete necropsy was done on all horses that died during the study, and representative samples of major organs and of the central nervous system were fixed in 10% neutral buffered formalin and processed routinely for histopathology.

Virology, Pathology and Serology

Representative portions of the brain and the entire spinal cord were submitted for virologic investigations. Part of the samples were frozen and sent for additional investigations to the Italian National Center for Exotic Diseases (Istituto Zooprofilattico Sperimentale Abruzzo e Molise, Teramo, Italy), the French National Reference Center for Arboviruses and Viral Haemorrhagic Fevers of the Pasteur Institute (Paris, France), and the International Reference Center for Borna Disease (Giessen University, Germany).

Two horses with neurologic signs were euthanized. A sample of cerebrum from one animal (no. 4083V) was analyzed in cell culture and by reverse transcriptase (RT)-PCR and semi-nested PCR. Samples of the cerebellum and the cervical, thorax, lumbar, and sacrum regions of the spinal cord from the second animal (no. 4553V) were also tested. The equivalent of about 0.5 mL of each tissue was mechanically crushed (Mini-beadbeater, Biospec Products, Inc., Bartlesville, OK) two times for 30 sec in 2 mL tubes containing 0.5 mL of sterile glass beads and 0.5 mL of Dulbecco's minimum essential medium (DMEM), supplemented with 10% fetal calf serum (FCS) and antibiotics, and centrifuged at 4,000 rpm for 15 min. The supernatant was diluted 1/10 and 1/100 in the medium and 0.2 mL of the three undiluted and diluted suspensions were incubated 1 hr at 37°C or 28°C in 24-well plates containing Vero E6 cells or *Aedes albopictus* C6/36 cells. After viral adsorption, cells were washed twice in DMEM and incubated 5 days in DMEM containing 3% FCS. On day 5, cell supernatants were used to infect cell monolayers in 25-cm^2 flasks and incubated for 5 additional days. Cells in each well and in the flasks were washed in phosphate-buffered saline (PBS), scraped with a rubber policeman, and deposited on immunofluorescent slides. The cells were fixed by air-drying, the membranes permeabilized in acetone, and dried. The cells were incubated for 30 min at 37°C with an appropriate dilution of hyperimmune mouse ascitic fluids prepared against several arboviruses causing encephalitis in horses. After washing in PBS, the bound antibodies were overlaid with an anti-mouse conjugate labeled with fluorescein. Slides were observed under fluorescent microscope.

RT-PCR and semi-nested PCR were performed on central nervous system samples, using a technique described previously (22). Briefly, total RNA was extracted from a volume of supernatant of ground central nervous system samples. Precipitated RNA was resuspended in diethyl pyrocarbonate–treated distilled water and subjected to RT and PCR, using the oligonucleotide primers (23) WN240 (5′ GAGGTTCTTCAAACTC-CAT 3′) and WN312 (5′ GAAAACATCAAGTATGAGG 3′).

One tenth of the incubation mixture was then re-amplified in a semi-nested PCR, using primers WN312 and WNEsn, (5′ CTCCA(T,G)GG(G,C)AGGTT(G,C)AG(G,A)TCCAT 3′). The presence of amplicons of 328 bp and 270 bp, respectively, were examined after 1.5% agarose gel electrophoresis and ethidium bromide staining. The sequences of the amplified products were characterized by sequencing genome position 1402-1656 of the envelope glycoprotein as described (23) by using the alignment algorithm Clustal W.

Serum samples from 12 of the 14 diseased horses were available; the remaining 2 horses had died without having blood samples taken. Additional samples were also available from the same premises where clinical cases had occurred and from nearby stables collected as part of the serologic survey described in the following section. Serum samples were first tested against the major neurotropic viruses infecting horses, including Eastern equine encephalomyelitis, Western equine encephalomyelitis, and Venezuelan equine encephalomyelitis. The presence of antibodies against WNV was evaluated using the CF test and by an immunoglobulin (Ig)G enzyme-linked immunosorbent assay (ELISA) technique.

Reagents for the CF test were supplied by the Onderstepoort Veterinary Institute, South Africa, and the Centers for Disease Control and Prevention, Division of Vector-Borne Infectious Diseases, Fort Collins, Colorado. The CF test assays were performed by the Italian National Center for Exotic Diseases according to the Office International des Epizooties procedures (24). The CF test was considered positive when the sample reacted at a titer of $\geq$1:4.

Crude antigens for ELISA were prepared from Vero E6 cells infected with the reference Egyptian Eg101 as described (25). Microtiter plates (Polysorb, Dynatech, Chantilly, VA) were alternately coated with 100 µL of WNV cell lysate antigen and with antigen derived from uninfected Vero cells. Plates were kept overnight at 4°C. Fourfold dilutions of horse sera, starting with 1:100, were placed in the wells. Bound IgG antibody was detected with goat anti-horse IgG conjugated to horseradish peroxidase. H_2O_2-tetramethylbenzidine was added and the optical density was measured at 420 nm. Serum samples were considered positive for corrected A_{420} values greater than the corrected mean A_{420} plus 3 standard deviations of four negative control sera tested at the same dilution.

Serologic Survey

After the initial notification of neurologic cases in horses, an area consisting of several administrative jurisdictions from which cases had been reported was delineated, and movement of horses in and out of the area was banned. The cause of the illness was not available at that time, and initial clinical and serologic investigations focused on stables located within a 3-km radius of those that had reported clinical cases.

By November 1998, when WNV was identified as the etiologic agent, we initiated a serosurvey to further define the geographic extent of the infection. The most peripheral stables within the restricted zone were used as reference points to construct a polygonal area whose external angles were all <180° (zone A). As shown in Figure 1, a second polygon was then drawn around the first, with all points 3 km distant from the internal polygon. Thus, a 3-km wide corridor between the first and second polygons was identified (zone B), all stables in this area were investigated, and blood samples from all horses were obtained. After identification of at least one seropositive horse in a stable located within the corridor, the polygon was redrawn with the new positive stable becoming a vertex of a new polygon and the corridor extended an additional 3 km (zone C). A total of 282 horse serologic data (IgG ELISA) were collected. Data were analyzed with the BMDP program version 1.0 (BMDP Statistical Software, Inc., Los Angeles, CA)

Results

Pathology

No gross changes were observed in the central nervous system and other organs of the six dead horses. Histologically, a mild-to-moderate nonsuppurative polioencephalomyelitis was present, with consistent involvement of the ventral horns of the thoracic and lumbar spinal cord and of the lower brain stem. Perivascular cuffs of lymphoplasmacytic and histiocytic cells with small and scattered glial nodules and focal gliosis were observed in the gray matter. In some cases, ring and petechial hemorrhages and neuronal degeneration were observed in the lumbar spinal cord (26). These features were highly suggestive of an infection of viral origin.

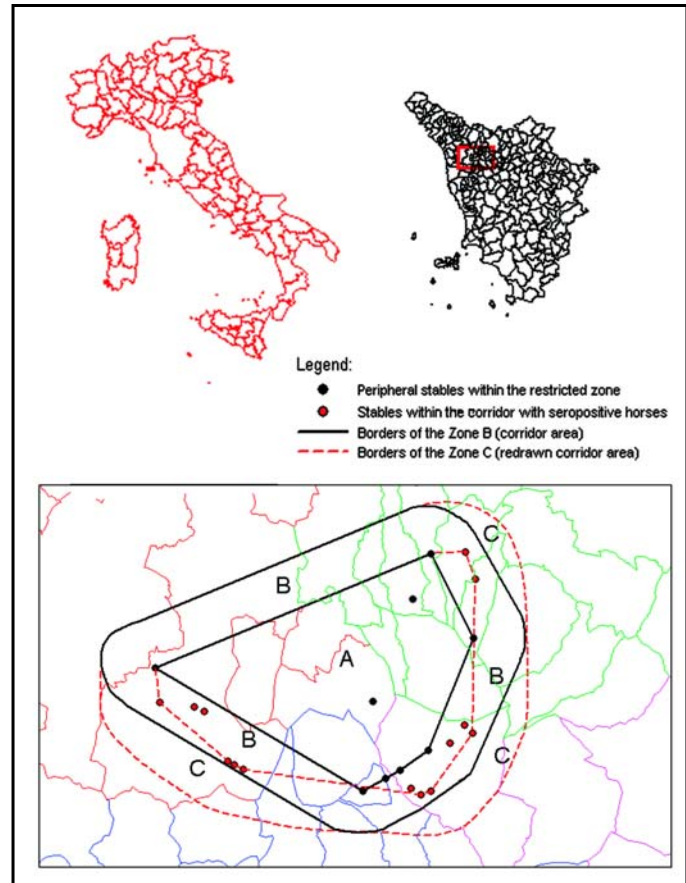

Figure 1. Map of the study area, showing the inner polygon within the restricted zone (zone A), the corridor area (zone B), and the redrawn corridor area (zone C), in the provinces of Firenze, Lucca, and Pistoia, Tuscany Region, Italy.

Virology

WNV was recovered in the cerebellum (Vero E6 and C6/36) (virus isolate PaAn981) and lumbar part (Vero E6) of the spinal cord of horse no. 4553V. RT-PCR was positive with samples from the cerebellum and the thorax and lumbar regions of the spinal cord. WNV was not isolated from the cerebrum of horse no. 4083V; however, RT-PCR results were positive. Both virus isolation and RT-PCR results were negative from the cerebrum of horse no. 4553V. In all cases, RT-PCR gave a negative result. Laboratory investigations on other viruses affecting horses yielded a negative result.

The sequence of the amplified fragment of 255 bp from the PaAn981 Italian virus RNA (GenBank accession no. AF205883) showed >99% similarity in nucleotide sequence with recent WNV strains responsible for epidemics in Romania in 1996 (GenBank accession no. AF260969), Morocco in 1996 (accession no. AF205884), Kenya in 1998 (accession no. AF146082), Volgograd in 1999 (accession no. AF239988, Israel in 2000 (accession no. AF380669), and France in 2000 (accession no. AF418554) and 98.6% similarity with strains from Israel in 1998 (accession no. AF205882) and New York in 1999 (accession no. AF196835). No change was observed in the amino acid sequence of this short region of the envelope protein.

Retrospective Study

The questionnaires sent to local veterinarians were returned by the end of October 1998. A total of 14 horses that matched the case definition were identified. They were distributed in nine different stables located in the provinces of Florence, Lucca, Pisa, Pistoia (Figure 1). The first case occurred on August 20 and the last on October 6. Of the 14 horses, 8 recovered, and 2 died 1 and 3 days, respectively, after onset of signs. The remaining four were euthanized because of the severity of the clinical course. The most frequently observed signs were posterior weakness, ataxia, and loss of equilibrium. In the eight animals that recovered, signs persisted for 5–15 days. In three cases, additional clinical signs were observed (mild keratitis, dermal papules, and third eyelid protrusion). Fever was observed in one case, at onset of disease.

Overall, the attack rate in the nine affected stables was 2.8% (14/498, Table 1). The case-fatality rate among horses with neurologic signs was 43% (6/14) including the four horses that were euthanized. No difference in age was detected between fatal cases (horses that died or were euthanized) and those that recovered (Mann-Whitney U Test).

The temporal distribution of equine cases by week of illness onset is shown in Figure 2.

On the 12 diseased horses for which blood was available for testing, CF test results were positive for all, with titers ranging from 1:4 to 1:128. In the nine stables from which clinical cases had been notified, 159 of the 498 horses present were bled in October and serologically tested, with 123 of 159 tested a second time 1 month later.

The overall results of the CF test are shown in Table 1. Table 2 shows the results of the first and second round of sampling in the 123 horses that were tested twice. The seroprevalence rate was significantly different between the two samplings (McNemar chi-square test; $p<0.01$). The 29 horses that were positive at first and second sampling had a geometric mean titer of 1:29 in the first sampling and 1:6 in the second sampling; this difference was statistically significant (Student t test for paired sera on log transformed data, $p<0.01$). Among the remaining 18 horses that initially tested positive but were negative at second testing, the geometric mean titer of the first sample was 1:15.

Table 1. Complement fixation test findings in stables with clinical cases (zone A)[a]

Stable	Horses present	Clinical cases	Seroprevalence	
			1st sampling[b]	2nd sampling[c]
1	25	2	11/21 (52.4%)	not available
2	5	1	3/5 (60%)	1/3 (33.3%)
3	12	2	6/10 (60%)	6/10 (60%)
4	49	2	9/13 (69.2%)	5/11 (45.4%)
5	7	1	2/7 (28.6%)	1/5 (20%)
6	270	1	5/49 (10.2%)	3/41 (7.3%)
7	52	1	9/20 (45%)	2/20 (10%)
8	18	1	6/8 (75%)	5/9 (55.5%)
9	60	3	12/26 (46.1%)	8/25 (32%)
Total	498	14	63/159 (39.6%)	31/124 (25%)

[a] Repeat titers (October–November 1998) were obtained from 123 horses.
[b] Performed October 12–16, 1998.
[c] Performed November 9–13, 1998.

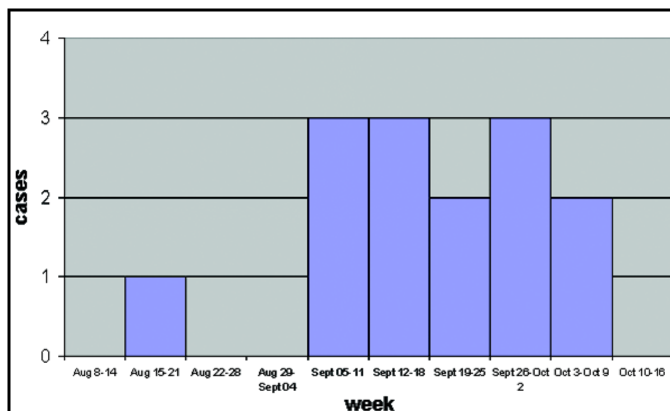

Figure 2. Number of neurologic cases in horses by week of onset, Valdinievole District, Tuscany, Italy, August–October 1998.

Serum samples were collected from an additional 161 horses in November 1998 (>3 weeks after the last reported clinical case) from 16 stables located within a 3-km radius from the ones with clinical cases in zone A. Samples were tested first with CF test and later with an IgG ELISA, not available at the beginning of the epidemic. Of the 161 horses, 63/155 (41%) had positive ELISA tests (6 horses were excluded because of inconclusive results) and 30/161 (19%) were CF test–positive with titers ranging from 1:4 to 1:8. The positive horses belonged to nine different premises. The overall seroprevalence rate in these nine stables was 49% (63/129) when the IgG ELISA test was used and 22% (30/134) with the CF test (Table 3).

To investigate whether rates of seropositivity differed between premises with and without clinical cases, the proportion of horses that were CF test–positive from the stables with clinical cases was compared with the proportion obtained for horses from the stables with silent infections. Because CF test results are likely to be dependent on the time of infection, the comparison consisted of the November samples from stables with silent infections and the second set of samples taken from horses from stables with clinical cases, also obtained in November (all stables from zone A). No significant difference was observed in rates of infection for the two groups (chi-square test $p>0.05$).

In February–April 1999, serum samples were obtained from all the 123 horses never tested before and housed in 35 stables located in zones B and C. Seroprevalence using the IgG ELISA test was 43/120 (36%), with three inconclusive tests. CF test prevalence was 19/123 (15%), with titers ranging from 1:4 to 1:8. Fourteen stables were found positive in zone B and none in zone C (Figure 1).

Among the 123 horses tested in zones B and C, information on presence in the area during the outbreak period was

Table 2. Comparison between first sampling (acute, October 1998) and second sampling (convalescent, November 1998) complement fixation test results in horses from the stables with clinical cases (zone A)

		1st sampling		
		Positive	Negative	Total
2nd sampling	Positive	29	1	30
2nd sampling	Negative	18	75	93
	Total	47	76	123

available for 110, including 107 that had been present and 3 that had not. Of the 107, 40 tested positive (37%); among the 3 not present, 1 was positive. Among the 13 horses whose location during the epidemic could not be established, 2 were seropositive.

The results of serum samples collected in zones B and C at the beginning of 1999 before the growth of the vector population were compared to the results of sera from the stables without clinical cases (zone A) collected during the autumn 1998. No differences were found between the percentages of animals seropositive by ELISA (36% vs. 41%) and CF test (15% vs. 19%). For this reason, to obtain a larger sample, all data were pooled to assess the age-specific prevalence in the horse population under study.

Table 3. Enzyme-linked immunosorbent assay (ELISA) and complement fixation (CF) test results in horses from stables with silent infection (zone A)

		ELISA		
		Positive	Negative	Total
CF test	Positive	26	4	30
CF test	Negative	37	88	125
	Total	63	92	155

A total of 282 horses were included in the analysis (Table 4) and classified according to age group and serologic status with respect to IgG ELISA (106 were positive; 9 were at the A420 cut-off value and inconclusive). No significant differences (chi-square >0.05) in the seroprevalence rate among the different age-classes were observed (Figure 3). The results of this survey confirmed a wider spread of infection, with an overall seroprevalence rate of 38% (106/282) in the affected area.

Discussion

An epidemic of WNV encephalitis occurred during the late summer of 1998, among horses residing in a wetland area in the Valdinievole Valley, in the provinces of Florence and Pistoia, Tuscany. All the cases identified by the retrospective study were confirmed by serologic, or virologic assays, or both.

The outbreak involved race horses and racehorse breeding stock of high economic value, and the spatial and temporal nature of this cluster aided the investigation, as did the fact that local practitioners brought the problem to the attention of veterinary public health authorities early in the the outbreak. The serologic survey demonstrated that WNV infection also occurred in premises without clinical cases and that WNV was spread over an area wider than initially detected during the epidemic.

Although the number of confirmed cases reported is small, the case-fatality rate of this 1998 Italian outbreak (6/14, 43%) is similar to the rate observed in 2000 in France (20/58, 34%), and in 1999 (23/60, 38%) and 2000 (9/25, 36%) in the United States (21,19). No differences in age were detected between fatal cases (horses that died or were euthanized) and those that recovered. Three of six fatal cases were observed in horses <6 years old, with one case 6 months old, one case 2 years old, and one case 5 years old. In the recent equine outbreak in France (21), >70% of the fatal cases occurred in horses in the 6- to 10-year (41.2%) and the 16- to 20-year (29.6%) age categories, with only one confirmed fatal case in the 1- to 5-year age category (5.9% of the fatal cases).

In our study, WNV was isolated from different parts of the central nervous system, and similar results were obtained in a horse with encephalitis euthanized during the epidemic in France in 1965 (27). In that case, WNV was isolated in cell cultures injected with material obtained from the lumbar region of the spinal cord but not with material from the cervical region of the spinal cord, the cerebrum, or the cerebellum. Although based on a very limited number of observations, we observed that the lower compartments of the brain and the spinal cord may be more infected than the cerebrum when the neurologic signs are predominant and the animal is dying. Moreover, the presence of neuronal necrosis in the brain in the absence of virus and the detection of both inflammatory lesions and virus in the lower brain stem late in the infection process suggest that the agent may have migrated from central brain areas downwards through the spinal cord (26). The low virus load in the central nervous system is in accordance with the small number of necrotic areas within the brain and the spinal cord tissues (3,26).

Two different serologic tests, which detect different classes of antibodies, can be used to distinguish between recent and past infections. CF test is used primarily to monitor IgM,

Table 4. Horses, classified by age class and serologic status (immunoglobulin G enzyme-linked immunosorbent assay), included in the analysis to assess age-specific prevalence[a]

Age class (yrs)	Horses tested	Inconclusive	Positive
0–2	45	3	17
2–4	63	1	21
4–6	49	1	18
6–8	30	1	10
>8	95	3	40
Total	282	9	106

[a]282 horses included because the age of 2 animals was unknown.

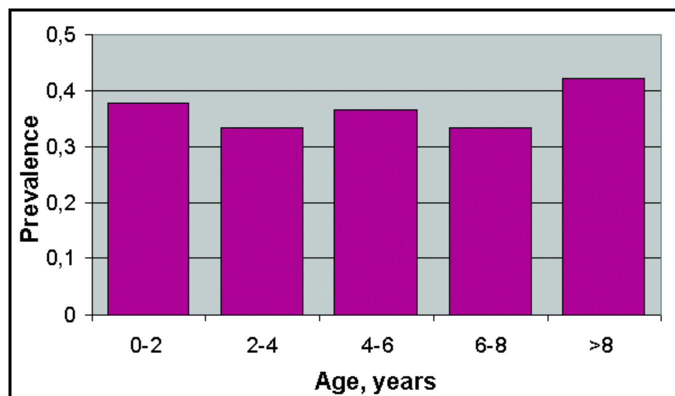

Figure 3. *West Nile virus* age-specific prevalence (n=282) in horses, Tuscany Region, 1998–1999.

although this test may not detect exclusively this class of antibodies, and the ELISA test is specific for IgG. The decline in the mean CF test antibody titer over a 1-month period (October–November 1998) in stables with clinical cases seems to be attributable to the natural decrease of CF antibody levels. The rapid decline in CF test titers may also explain the lower proportion of CF test–positive horses in the second and third groups that were tested in November and in early 1999. In the zone A stables, which had silent infections and that were investigated in November 1998, the CF test prevalence rates were similar to the rates detected in the November samples taken from the neighboring stables with clinical cases. As similar IgG ELISA prevalence rates were obtained both in these stables with silent infections located in zone A and in stables in zone B (sampled in early 1999), we assume that, apart from the observation of neurologic signs, all horses residing in the study area had the same level of exposure to the virus.

Retrospectively, serologic results suggest that the epidemic ended during the autumn of 1998, probably because further major circulation of virus was greatly reduced by the effect of the low temperature on the population density of mosquitoes. However, at present no information is available regarding the vector species involved in this outbreak.

The seasonal distribution we observed is in keeping with other studies. All previous equine and human outbreaks of WNV infection in Europe and the Mediterranean area typically have occurred between August and October (9,21), the period when the population density of culicine mosquitoes is highest. Similar observations were made in the United States in the equine outbreaks in 2000 (19), with cases identified in seven northeastern states situated at latitudes similar to that of central and northern Italy.

The differences among age-specific prevalence rates in the whole area under investigation were not significant, indicating that the horse population in the area under study had not been exposed in the previous years to WNV. The most probable hypothesis is that the virus was introduced in the Padule del Fucecchio wetlands by migratory birds during the spring 1998. Migratory birds such as storks may play an essential role in the introduction of WNV when they land in wetlands with high levels of ornithophilic mosquitoes (28). The circulation of a unique genotype in Italy in 1998, Morocco in 1999, and France in 2000 suggests that migratory birds crossing the Mediterranean Sea could be the common cause of virus emergence. The 1998 WNV outbreak in Italy was not preceded by notification of any significant deaths among wild avian species. Indeed, natural illness and death of wild birds resulting from WNV had never been reported before the 1998 episode in storks in Israel (28) and the 1999 epidemic in birds reported in the northeastern United States. Old World wild avian species may have developed co-evolutionary adaptation to various WNV genotypes; the recent North American epidemic could have occurred because the lack of avian adaptation or the greater virulence, especially for *Corvidae* (29), of the strain involved. The recent European WNV outbreaks were apparently not associated with bird deaths (5,21,15). The comparison of the amino acid sequence of the entire envelope protein among European WNV isolates and strains from Israel 1998 and New York 1999, known to be significantly pathogenic for some species of birds (30), showed two amino acid changes that are attenuating mutations for other flaviviruses of the Japanese encephalitis group (31). Whether viral genetic factors or ecologic factors are responsible for apparent differences in virulence for birds, horses, and humans remains unknown and merits further multidisciplinary investigation.

In 1999 and 2000, no cases of neurologic disease were recorded in horses in Tuscany, and no significant wild bird deaths or rise in human neurologic cases were detected. Unfortunately, conducting further studies to determine if any viral activity was still present in the area was not possible. The risk that WNV could remain endemic in the area because of transovarial transmission or overwinter survival of mosquitoes cannot be ruled out. However, at least in temperate regions of Eurasia, the usual pattern of WNV epidemics may be the result of virus importation by birds during their migration north to their breeding grounds (32). Still, outbreaks of WNV infection are a relatively rare event in countries where populations of wild birds regularly migrate every year from endemic areas. The reason may be that the emergence of a mosquito-borne infection always involves a series of particular ecologic conditions, including seasonal environmental factors, presence of infectious migrating hosts, ornithophilic vectors, amplifying avian hosts, and susceptible accidental hosts in the same geographic area.

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Induction of Inflammation by *West Nile virus* Capsid through the Caspase-9 Apoptotic Pathway

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West Nile virus (WNV) is a member of the *Flaviviridae* family of vector-borne pathogens. Clinical signs of WNV infection include neurologic symptoms, limb weakness, and encephalitis, which can result in paralysis or death. We report that the WNV capsid (Cp) by itself induces rapid nuclear condensation and cell death in tissue culture. Apoptosis is induced through the mitochondrial pathway resulting in caspase-9 activation and downstream caspase-3 activation. Capsid gene delivery into the striatum of mouse brain or interskeletal muscle resulted in cell death and inflammation, likely through capsid-induced apoptosis in vivo. These studies demonstrate that the capsid protein of WNV may be responsible for aspects of viral pathogenesis through induction of the apoptotic cascade.

West Nile virus (WNV) is a member of the *Flaviviridae* family, which includes *St. Louis encephalitis virus*, Kunjin virus, *yellow fever virus*, *Dengue virus*, and *Japanese encephalitis virus* (1). WNV, a single-stranded RNA virus, was initially isolated in the West Nile region of Uganda in 1937 (1) and has become prevalent in Africa, Asia, and Europe. Since its introduction into the United States in summer 1999, the sudden and rapid spread of this virus in the United States has caused much concern. WNV has been reported in infected mothers' breast milk, and WNV transmission by organ transplantation and transfusion has been documented. Clearly, WNV infection is not only a regional public health problem, but a global health issue (2). However, we lack a clear understanding of WNV pathogenesis, and little specific treatment exists for WNV infection. Therefore, a clearer understanding of WNV is necessary in order to identify new strategies to treat or prevent this viral infection (3).

Here we report on an unexpected role for WNV capsid (Cp) in viral-induced pathogenesis. We observed that the WNV-Cp protein is a pathogenic protein, which drives apoptosis in vitro through the mitochondrial/caspase-9 pathway. We also observed that expression of Cp protein in mouse muscle resulted in apoptosis and inflammation of muscle cells. More importantly, direct in vivo expression of WNV-Cp protein in mouse brain resulted in an induction of apoptosis similar to what is observed in natural infection. These results provide evidence of a link between WNV-Cp protein and WNV pathogenesis in vivo.

Materials and Methods

Cloning and Expression Analysis of WNV-Cp Gene

The cloning of a synthetic WNV-Cp gene based on the reported NY-99 infectious strain was described earlier (4). Western blot analysis was performed as previously described (4). For a caspase-9–specific test, 5 µg of pcWNV-Cp-DJY or pcWNV-CpWT was cotransfected with a dominant negative caspase-9 (DN caspase-9) construct, and cleavage of procaspase-9 protein was determined by Western blot analysis with antihuman caspase-9 antibody (MBL, Nagoya, Japan). DN caspase-9 (provided courtesy of Emad S. Alnmeri, Thomas Jefferson University, Philadelphia, PA) has been reported to inhibit the caspase cascade (5). The localization pattern of capsid expression was analyzed by immunofluorescent assay in HeLa, 293-T, RD, or SH-SY5Y cells by using anti-His tag antibody as described (6).

Observations with Electron Microscope

RD cells transfected with pcWNV-Cp-DJY or pcDNA3.1 plasmid DNA were processed for transmission electron microscope analysis as described (7,8). Semithin (1.0-µm) sections were stained with toluidine blue, and photographed with Ektachrome 160T film (Eastman Kodak Co., Rochester, NY). Ultrathin sections were stained with uranyl acetate and lead citrate, and observed with a Philips CM-100 electron microscope, operated at 60 Kv.

TUNEL Assay and Annexin V Staining

In vitro apoptosis in individual cells was determined by terminal desoxyriboxyl-desoxyriboxyl transferase–mediated

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DVTP nick-end labeling (TUNEL) assay with the In Situ Cell Death Assay Kit (Roche Diagnostic Corp., Indianapolis, IN) and visualized by fluorescent microscopy. Apoptosis induction by the expression of capsid was also determined by annexin V staining procedure followed by fluorescence-activated cell sorter analysis. Cells were transfected with the WNV-Cp–enhanced green fluorescent protein (EGFP) fusion construct or pcDNA3.1. Forty-eight hours after transfection, the cells were stained with phycoerythrin-conjugated annexin V. Only EGFP-expressing cells were analyzed and the data were acquired by using the CellQuest software package (Becton-Dickinson, and Co., Franklin Lakes, NJ).

Mouse Muscle Injection

Female 6- to 8-week-old Balb/c mice (Charles River Laboratories, Inc., Wilmington, MA) were injected in the tibialis muscle with 100 µg of pcWNV-Cp-DJY or pcDNA3.1 in phosphate-buffered saline (PBS) and 0.25% bupivicaine-HCl (Sigma-Aldrich Corp., St. Louis, MO) as described (9). After 48 h, the tibialis muscle was harvested and embedded in OCT Compound (Sakura Finetek U.S.A., Inc., Torrance, CA). Muscle sections were prepared by cryosectioning and stored at –20°C until assayed. For pathologic observation, tissue sections were stained with hematoxylin/eosin (9).

DNA Injection into Mouse Brain

Balb/c mice were anaesthetized with ketamine/xylazine (70 mg/kg of ketamine, 7 mg/kg of xylazine). Using a Hamilton syringe (Hamilton Co., Reno, NV) with a 30-gauge removable needle, 5 µg of pcWNV-Cp–DJY or pcDNA3.1 DNA, in 5 µL of endotoxin-free water and 0.25% of bupivicaine-HCl in PBS was injected into the frontal cortex (striatum) with a small animal stereotactic apparatus (Kopf Instruments, Tujunga, CA) as described (10). The DNA was injected for 3 min; the needle was left in the place for 1 min and then withdrawn slowly over 1 min.

Mouse Brain Tissue Immunohistochemistry by Using Horseradish Peroxidase (HRP)

Twenty four to 48 h postinjection, mice were deeply anesthetized and perfused transcardially with 0.1 M PBS (pH 7.2), then with 4% paraformaldehyde (PFA) in PBS. The brains were postfixed in 4% PFA for 18 h at 4°C and cryoprotected in 30% sucrose for 48 h at 4°C, then frozen and mounted for cryostat sectioning. Sections (25 µm) were serially cut in the coronal plane. The tissue sections were treated with anti-Histag antibody with appropriate secondary antibody with the counterstaining of hematoxylin. The slides were analyzed under a fluorescent microscope for TUNEL or gene expression.

Detection of Mictochondria-Based Apoptotic Pathways

Caspase-3 (Pharmingen, San Diego, CA), caspase-8 (FADD-like interleukin-1 beta-converting enzyme) and caspase-9–like Mch6 (MBL, Nagoya, Japan) activities were determined according to the manufacturer's protocol. RD cells transfected with pcWNV-Cp–DJY or pcDNA3.1 were harvested and lysed at 48 h postinjection. The cell lysates (100 µg/100 µl protein) were incubated with specific substrate Ac-DEVD-AMC for caspase-3, IETD-*p*NA for caspase-8, or LEHD-*p*NA for caspase-9 for 1–2 h at 37°C. For the inhibition test, IETD-FMK or LEHD-FMK, inhibitors for caspase-8 or -9, respectively (MBL) were added to the reaction, along with the substrate, according to the protocol. The activity of released AMC or *p*NA was determined by spectrophotometer at 405 nm. The mitochondria transmembrane potential was measured by using a DePsipher assay kit (R&D Systems, Minneapolis, MN). The cells were observed under a fluorescent microscope, and the images were acquired and analyzed in an Image-Pro program (Media Cybernetics, Inc., Houston, TX).

Results and Discussion

WNV is a vector-borne pathogen that induces encephalitis and death in WNV-endemic regions (11). Unfortunately, no specific therapy exists for WNV infection (3). Furthermore, the exact mechanisms of WNV-induced pathogenesis have not been elucidated. To attain a better understanding of possible mechanisms of WNV biology, we studied the role of the capsid gene in WNV pathogenesis.

WNV-Cp Protein Induces Apoptosis in Cells In Vitro

The expression of the Cp gene was examined by in vitro transcription/translation system as well as Western blot analysis (Figure 1a-h). The cells expressing WNV-Cp, as well as the positive control plasmid encoding the proapoptotic protein, Bax, show nuclear condensation, which is a typical feature of apoptotic cells. We carried out TUNEL assays for apoptosis with a double-staining procedure. The WNV-Cp–transfected HeLa cells were simultaneously stained for TUNEL assay, which reveals nuclear condensation, and for capsid expression with rhodamine-conjugated secondary antibody. The double-positive cells (Figure 2i) (Figure 2j) indicate the induction of apoptosis specifically driven by the expression of capsid.

Cells transfected with pcWNV-Cp-DJY were further investigated by transmission electron microscopy. Examination of semithin sections (1.0 µm) stained with toluidine blue revealed typical apoptotic cells representing approximately 5% of the total cell population in pcWNV-Cp-DJY–transfected cells (Figure 2d, arrow) but not in control cells (Figure 2e). These apoptotic cells usually lost their polygonal shape as well as their contact with neighboring cells, and became round and stained exceptionally dark. Many of the apoptotic cells also exhibited clear vacuoles in the condensed cytoplasm. Fragmented, but equally condensed, apoptotic bodies were also present. Ultrastructurally, all of the apoptotic cells showed continuous plasma membranes, apparent aggregation of nuclear chromatin, highly condensed cytoplasm, and nearly intact organelles (Figure 2f). Cells transfected with pcWNV-Cp-DJY show typical features of apoptosis.

The induction of apoptosis by capsid expression was also confirmed in different human cell lines such as HeLa, 293, RD, or SH-SY5Y. All three cell lines, HeLa, 293, and RD, transfected with pcWNV-Cp-DJY were TUNEL-positive (Figure 2g, 2i, and k, respectively), whereas the control transfected cells were not (Figure 2m). Proapoptotic Bax expression plasmid was used as a positive control. Experiments with annexin V staining revealed that 22.9% of WNVCp-transfected cells undergo phosphatidylserine dislocalization, a typical early feature of apoptosis (Figure 2o).

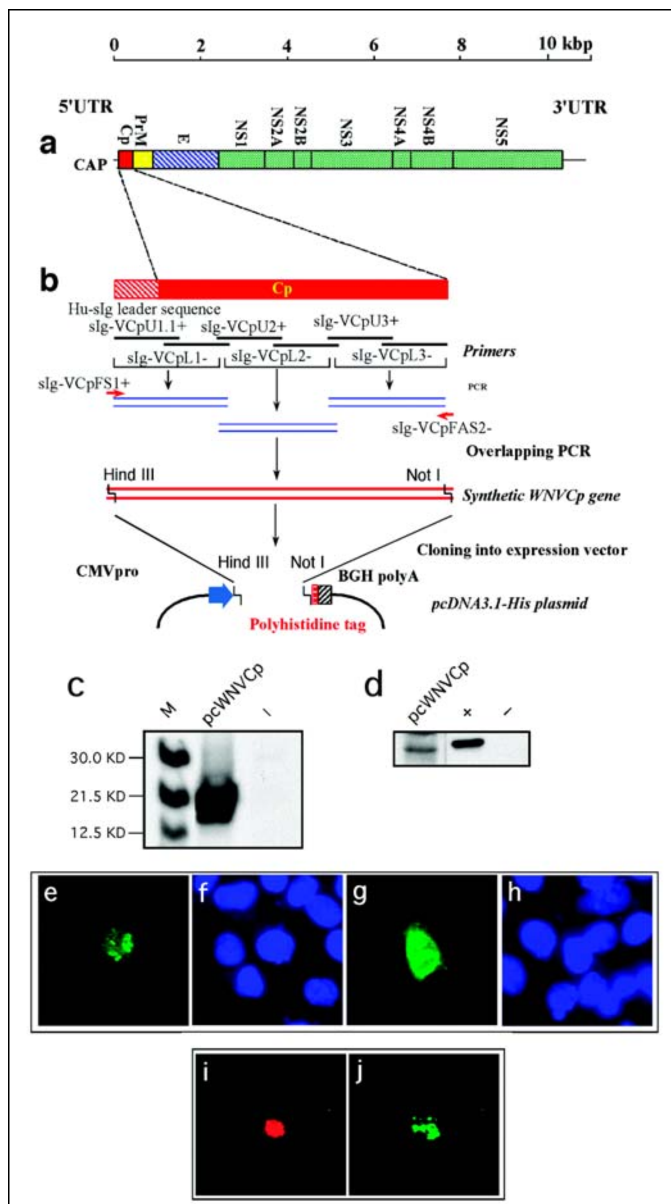

Figure 1. Construction and subcellular expression of *West Nile virus* (WNV)–NY1999 capsid (Cp) gene–expressing plasmid, pcWNV-Cp-DJY: a, Genomic organization of WNV-NY1999 (10,945 bp) is outlined based on the published (GenBank accession no. AF202541). b, Cloning strategy for WNVCp gene–expressing plasmid, pcWNV-Cp-DJY. c, In vitro translated and immunoprecipitated ^{35}S-labeled WNV-Cp visualized by SDS-PAGE. WNV-Cp–specific protein synthesis was compared to control generated by the vector backbone pcDNA3.1 (-). d, Protein expression by Western blot analysis, of WNV-Cp expression in HeLa cells. Subcellular location of WNV-Cp protein, in HeLa cells transfected with pcWNV-CpWT (e,f) or pcWNV-Cp-DJY (g,h) plasmids. 16 h post-transfection, the cells were visualized by indirect immunofluorescence. Typical nuclear staining was observed with the cells expressing WNV-CpWT (e) compared to the cells expressing WNV-Cp-DJY (g). TUNEL assay on the WNV-Cp–transfected cells, indicating nuclear condensation (i) due to expression of capsid (j).

Apoptosis by WNV-Cp In Vivo

We have shown, as have other researchers, that induction of apoptosis by plasmid in vivo results in enhanced levels of proinflammatory T-cell activation (4,9,12–14). We extended these in vitro findings of the ability of WNV-Cp to induce apoptosis to an animal model in vivo by using a direct plasmid delivery method as described (9,14). At 24 h after plasmid injection, TUNEL revealed positive signals (noted by dark brown because of the HRP-DAB reaction) in pcWNV-Cp-DJY injected mouse muscle (Figure 3a, arrows) but not in control muscle (Figure 3b). We observed severe inflammation within 48 h in mouse muscle injected with pcWNV-Cp-DJY (Figure 3c) but not in the pcDNA3.1-injected mouse muscle (Figure 3d). These studies indicate that expression of Cp protein in mouse muscle resulted in apoptosis and inflammation of muscle cells in vivo. In this regard, induction of apoptosis and the resulting inflammatory cell infiltration induced by WNV-Cp expression may have an important role in viral pathogenesis.

WNV has been found in the brains (15) and cerebrospinal fluids (16) of infected patients, where it induces cell death, resulting in encephalitis (17–19). As no component of WNV had been previously implicated in in vivo cell death of neuronal tissue, we reasoned that the WNV-Cp was a possible candidate, and we sought to investigate the effects of WNV-Cp in the brain in vivo. We directly injected DNA into the brain because that approach would not be complicated by vector delivery.

Mice were injected stereotactically with WNV-Cp or control plasmid DNA and euthanized 24–48 h after injection. Sections were processed from the harvested brain samples as described in Materials and Methods. By using a monoclonal antibody specific to the His epitope contained in the plasmid vector, immunohistochemical analysis revealed that His-positive cells, as identified by HRP or fluorescein isothiocynate (FITC) were found in pcWNV-Cp-DJY–injected mouse brain (Figure 3f, h, respectively). Similar expression was absent in the brains of mice injected with control plasmid (Figure 3e, j, respectively). However, His-positive cells were detected in several areas of the cerebral cortex, including the motor cortex of the pcWNV-Cp-DJY injected mice (Figure 3f by HRP, and 3h by FITC [arrows]). Nuclear condensation, a classic feature of apoptosis, was also observed in these sections by DAPI staining (Figure 3i and 3m; arrows). As shown in Figure 3g by HRP and 3l by FITC, we observed TUNEL-positive cells in the brain sections from the pcWNV-Cp-DJY–injected mice (Figure 3g and 3l) and not in the pcDNA3.1–injected control mice (Figure 3n). These TUNEL-positive cells were localized to the specific sites of injection (Figure 3l, arrow). These results illustrate that His-expressing cells were also TUNEL-positive, showing the direct relationship of WNV-Cp expression and in vivo apoptosis. WNV-Cp protein expression and

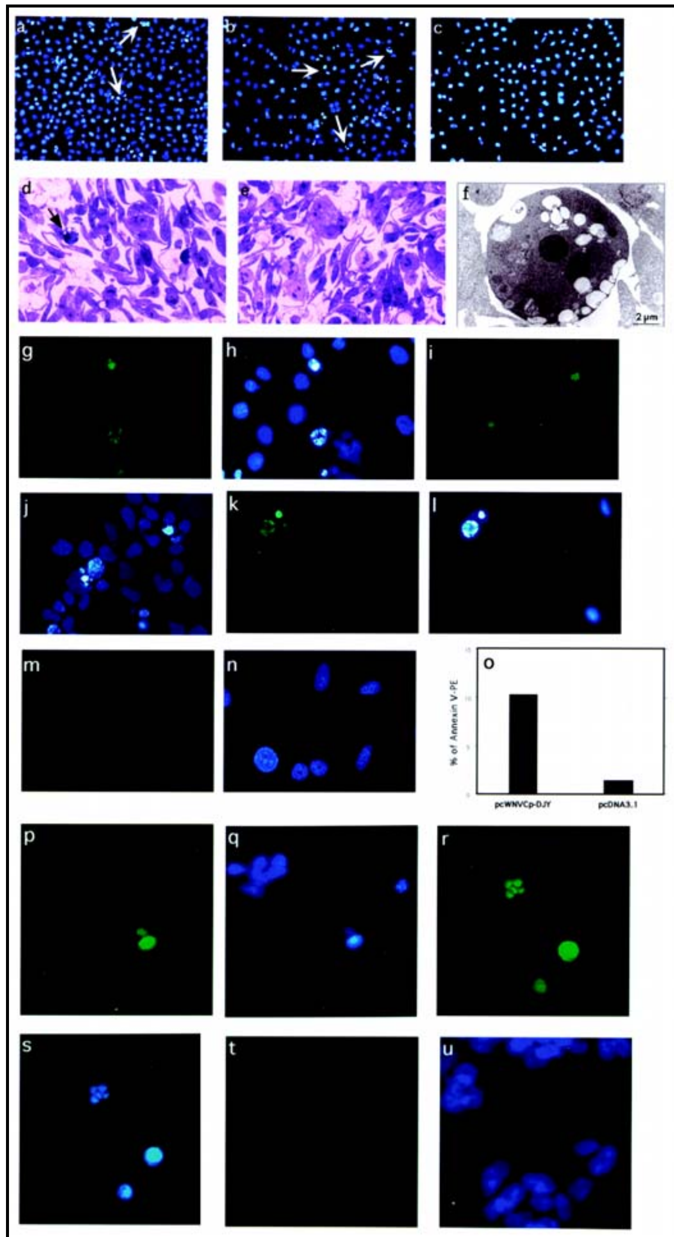

Figure 2. *West Nile virus* capsid (WNV-cp)-DJY protein expression induces apoptosis. Nuclear condensation was observed in HeLa cells transfected with pcWNV-Cp-DJY (a), a positive control pBax (b), or a negative control, pcDNA3.1 (c), under a 4,6-diamidino-2-phenylidole (DAPI) filter (magnification: 200X). Light microscopic observation on pcWNV-Cp-DJY (d) or pcDNA3.1 (e) plasmid transfected RD cells were examined in semithin sections stained with toluidine blue (magnification for d and e: 400X). Ultramicroscopic image of apoptotic cells were photographed from pcWNV-Cp-DJY transfected RD cells (f). DNA fragmentation in WNV capsid–expressing cell lines was examined by terminal desoxyriboxyl-desoxyriboxyl trasferase–mediated DVTP nick-end labeling (TUNEL) assay in HeLa (g), HEK 293 (i), and RD cells (k), and compared with DNA fragmentation from pcDNA3.1-transfected HeLa cells (m). Nuclear staining in HeLa (h), HEK 293 (j), and RD cells (l) were observed by using a DAPI filter and compared with control HeLa cells (n) (magnification for g through n: 400X). Cell membrane morphology changes were examined by annexin V staining/flow cytometry by using HeLa cells transfected with pcWNV-Cp-DJY or control pcDNA3.1 plasmids (o). The human neuroblastoma cell line SH-SY5Y was transfected with Bax as a positive control (p), pcWNV-Cp (r), or control plasmid (t) and examined by TUNEL assay. To visualize nuclear staining, cells transfected with pBax, pcWNV-Cp-DJY (q and s, respectively) or pcDNA3.1 (u) were stained with DAPI and observed using appropriate filters (magnification: 400X).

apoptosis as well as inflammation were highly reproducible in all animals studied by injection with pcWNV-Cp-DJY plasmid. These data strongly suggest that the expression of the WNV-Cp protein in the central nervous system can play a role in neuronal cell death, and this process may be important in the pathogenesis of WNV-induced encephalitis.

Mitochondrial-Activated apoptotic Pathway

To characterize the apoptosis pathway activated by the WNV-Cp protein, we next examined its direct effects on the disruption of the mitochondrial transmembrane potential in these cells. HeLa-CD4 cells were transfected with pcWNV-Cp-DJY or pcDNA3.1 plasmids, and the mitochondrial membrane potential was measured with a DePsipher assay kit (R&D Systems). The pcDNA3.1-transfected cells showed a normal pattern of orange-red fluorescence (Figure 4b). In contrast, green fluorescence was clearly visible in the pcWNV-Cp-DJY–transfected cells (Figure 4a, arrows).

We examined the effects of WNV-Cp on caspase-3, -8, or -9 activity. Cell lysates from pcWNV-Cp-DJY–transfected cells showed marked activity for caspase-3, illustrating substantial apoptotic induction (Figure 4c). Moreover, the cell lysate harvested from pcWNV-Cp-DJY–transfected cells was positive for caspase-9 activity, and this activity was inhibited by the addition of the caspase-9-specific inhibitor, LEHD-FMK (Figure 4d). In contrast, caspase-8 activity from these samples was not greatly increased relative to the negative control, and little effect was noted by the addition of the caspase-8–specific inhibitor, IETD-FMK (data not shown). Cell lysates from pcDNA3.1 control transfected cells show no caspase-8 (data not shown) or -9 activity (Figure 4d). These results firmly suggest that the mechanism of WNV-Cp–induced apoptosis is through the disruption of the mitochondrial transmembrane potential and the activation of caspase-9, which result ultimately in activation of the caspase-3 pathway.

Mapping Apoptosis-Inducing Domain

To map the apoptosis-inducing domain, a 3′-terminal deletion mutant with deletion of 3′-terminal 55 amino acids was generated, and its integrity was tested by in vitro translation/transcription (Figure 5a,b). Furthermore, to examine whether the specific 3′-terminal region is a determinant for the observed apoptosis, plasmids were transfected into RD cells, and cell lysates were analyzed for caspase-3, -8, and -9 activities. The native WNV-Cp constructs showed strong caspase-3 activity (Figure 5c). In addition, this 3′-deletion mutant showed similarly lower induction of caspase-9 activity (Figure 5d). These data support the hypothesis that this 3′ domain plays an important role in the induction of apoptosis by the WNV-Cp protein. Furthermore, to confirm that this apoptosis-induction pathway is through caspase-9, a domant negative (DN) caspase-9 construct, which has been reported to inhibit the caspase cascade, was cotransfected wiwth pcWNV-Cp-DJY or pcWNV-CpWT and the expression level of pro-caspase-9 cleavage products (35–37 kDa) was compared to the

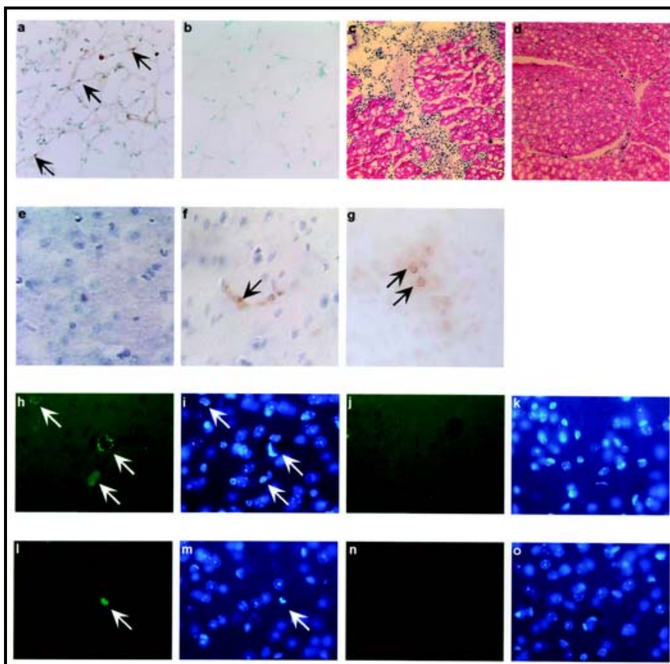

Figure 3. In vivo *West Nile virus* capsid expression induces apoptosis and inflammation in mice. TUNEL assay was performed on muscle cryosections harvested from mice injected with pcWNVCp-DJY (a) or pcDNA3.1 (b). Hematoxylin/eosin staining was performed on mouse tibialis muscle cryosections harvested from mice injected with pcWNVCp-DJY (c) or pcDNA3.1 (d) at 48 h postinjection (magnification: 200X [a, b] and 40X [c, d]). Immunohistochemical analysis was performed for detection of WNVCp-DJY protein expression in mouse brain injected with pcDNA3.1 or pcWNVCp-DJY as detected with HRP [Q17: spell out?](e, f, respectively). TUNEL assay on mouse brain cryosections harvested from pcWNVCp-DJY injected mouse was detected with HRP (g) (magnification: 300X [e through g]). Immunohistochemical studies were performed for detection of WNVCp-DJY protein expression in mouse brain injected with pcWNVCp-DJY or pcDNA3.1 as detected by fluorescein isothiocynate stain (h, i, and j , k, respectively). TUNEL assay on mouse brain cryosections harvested from pcWNVCp-DJY–injected mice (l, m) or pcDNA3.1-injected mice as detected with fluorescein isothiocyanate (n, o). WNVCp-DJY protein expressing His-positive cells or TUNEL-positive cells were visualized under ultraviolete microscope (h or l, respectively). Nuclear staining for WNVCp-DJY– or pcNDA3.1-transfected cells was visualized with appropriate filters (i, m or k, o, respectively) (magnification: 630X [h through o]).

activity of the 3′-deletion mutant, pcWNV-CpΔ3′ and pcDNA3.1 in Western blot analysis. The DN caspase-9 specifically blocked the cleavage of pro-caspase-9 in pcWNV-Cp-DJY and pcWNV-CpWT cotransfected cell lysates compared to those of pcWNV-Cp-DJY or pcWNV-CpWT in transfected cell lysates (Figure 5e). Moreover, the cell lysate transfected with the 3′-terminal deletion mutant had lower cleavage of pro-caspase-9, which is related to the lower induction of caspase-3 and –9 activity as determined by protease activity assay (Figure 5c,d). Cell lysates from pcDNA3.1 control transfected cells show much less pro-caspase-9 cleavage products.

Although the apoptotic effects of wild-type WNV as well as other flaviviruses have been previously reported, the gene or genes responsible for this effect in WNV have not been described. The Cp-induced apoptosis in the brain implies that the expression of the WNV-Cp protein in the central nervous system may play an important role in initiating neuronal cell death through apoptosis-induced inflammation. Therefore, this process may be important in the pathogenesis of WNV-induced encephalitis.

In this study, the WNV-Cp protein–induced apoptosis through the destabilization of the mitochondrial transmembrane, resulting in the likely release of cytochrome c (20,21). The complex of cytochrome c/Apaf-1 recruits and activates procaspase-9 (22), not procaspase-8. Paradoxically, the karyophilicity of WNV-Cp protein does not fully explain the destabilization of the mitochondrial membrane and its ability to drive the caspase-9 apoptotic pathway. Therefore, it is possible that WNV-Cp changes the host cell transcriptional machinery, resulting in an over expression of certain proteins related to an apoptotic program, which consequently feed back to the mitochondria, or that as WNV-Cp moves from the cytoplasm to the nucleus, it may sequester or inactivate an important member of the antiapoptotic pathway or the cell cycle pathway, and thus induce the apoptotic cascade. Furthermore, the data suggest that WNV-Cp may interact with host cell proteins to induce apoptosis in the host cell. Identifying these proteins will likely give more insight into the biology of WNV. This biology likely involves the WNV-Cp 3′ region. Moreover, this flavivirus contains a capsid protein, which localizes to the nucleus. Flavivirus replication is normally cytoplasmic, although some evidence supports nuclear function as part of the viral life cycle. In fact, Kunjin virus capsid has been found in the nucleus (23). Our results identify the nuclear localizing property of the protein as a potential pathogenic attribute. Hence, the pathogenic region of the protein is localized within the 3′-terminal region. Therefore, creating a WNV isolate that no longer localizes the capsid to the nucleus may result in a virus that loses pathogenesis, providing a novel approach for

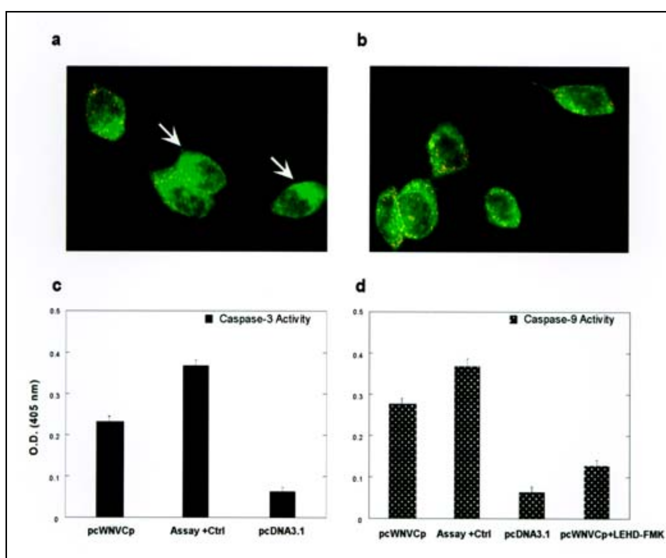

Figure 4. Mitochondria transmembrane potential and caspase activities measurement. HeLa-CD4 cells were transfected with pcWNVCp-DJY (a) or pcDNA3.1 (b), and their mitochondria transmembrane potential was measured after 48 h by ultraviolete illumination. A colorimetric caspase activity assay was performed with pcWNVCp-DJY– or pcDNA3.1-transfected cells for caspase-3 (c) or caspase-9 (d) activity. As a specificity control, the inhibitor LEHD-FMK for caspase-9 was added to the reactions along with relevant substrate (d). A specific positive control was used for assay validation (magnification: 1000X [a and b]).

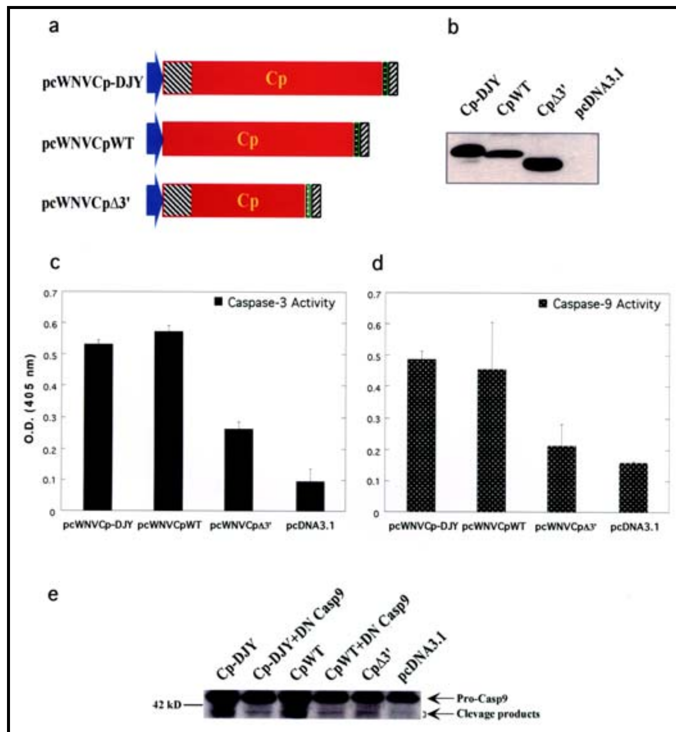

Figure 5. Apoptosis determining domain in WNVCp gene. a, Schematic diagram of pcWNVCp-DJY, pcWNVCpWT and pcWNVCp_3’ constructs. b, Immunoprecipitation of in vitro translated protein from pcWNVCp-DJY (Cp-DJY), pcWNVCpWT (CpWT), and pcWNVCp-3’ (Cp_3’) plasmids. As a negative control, pcDNA3.1 in vitro translated supernatants were analyzed. c, Colorimetric caspase-3 activity assay using pcWNVCp-DJY (Cp-DJY), pcWNVCpWT (CpWT), or pcWNVCpD3’ (CpD3’) plasmid transfected cells. d, The cell lysates were assayed for caspase-9-like activity, and the pcDNA3.1 transfected cell lysate was used as the negative control. e, Inhibition of WNVCp induced apoptosis by a dominant negative (DN) caspase-9 plasmid (DN Casp9) was assayed with equal amount of cell lysates from co-transfection of pcWNVCp-DJY (Cp-DJY) or pcWNVCpWT (CpWT), an expression level of pro-caspase-9 cleavage products (35 to 37 kDa) was compared to 3′-terminal deletion mutant, pcWNVCp_3’ (Cp_3′) and pcDNA3.1 by Western blot analysis with anti-human caspase-9 mAb.

vaccine studies. These results also imply that inhibiting the C-terminal region’s ability to interact with its putative ligand could be an important target for the development of new treatments for WNV infection.

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Vector Competence of California Mosquitoes for *West Nile virus*

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To identify the mosquito species competent for *West Nile virus* (WNV) transmission, we evaluated 10 California species that are known vectors of other arboviruses or major pests: *Culex tarsalis, Cx. pipiens pipiens, Cx. p. quinquefasciatus, Cx. stigmatosoma, Cx. erythrothorax, Ochlerotatus dorsalis, Oc. melanimon, Oc. sierrensis, Aedes vexans,* and *Culiseta inornata*. All 10 became infected and were able to transmit WNV at some level. *Ochlerotatus*, *Culiseta*, and *Aedes* were low to moderately efficient vectors. They feed primarily on mammals and could play a secondary role in transmission. *Oc. sierrensis*, a major pest species, and *Cx. p. quinquefasciatus* from southern California were the least efficient laboratory vectors. *Cx. tarsalis, Cx. stigmatosoma, Cx. erythrothorax,* and other populations of *Cx. pipiens* complex were the most efficient laboratory vectors. *Culex* species are likely to play the primary role in the enzootic maintenance and transmission of WNV in California.

Three years since its 1999 introduction into North America, *West Nile virus* (WNV) has spread rapidly from New York to the Rocky Mountains and to the Gulf of Mexico. As of September 2002, over 1,900 human cases of WNV encephalitis have been confirmed with 94 deaths; >6,000 equine cases also occurred during 2002 (1). The imminent spread of this virus culminated in the establishment of WNV surveillance programs in 48 states. Surveillance programs include testing mosquito pools for virus, sentinel chickens for seroconversion, wild birds for virus and seroconversion, and equine and human cases (2).

WNV is a geographically widespread arbovirus in the family *Flaviviridae*, genus *Flavivirus* (3). The virus, first isolated from the blood of a woman in the West Nile district of Uganda in 1937 (4), historically has been endemic to Africa, Western Asia, and the Middle East. Recently, WNV has expanded its distribution and caused epidemics in Russia, Romania, France, and Israel (5,6).

WNV is maintained in an enzootic transmission cycle among *Culex* mosquitoes and wild birds. In Africa and the Middle East, WNV has been most frequently isolated from *Cx. univitattus* (7,8). In Asia, members of the *Cx. vishnui* complex have been implicated as the primary vectors (9). *Cx. modesuts* was identified as a principal vector during a 1960s epidemic in France (3). During the North American outbreak, members of the *Cx. pipiens* complex were considered the primary epizootic vectors (10). Since the New York outbreak in 1999, WNV has been recovered from 26 North American mosquito species, including *Cx. pipiens*, *Cx. salinarius*, *Cx. restuans, Ochlerotatus canadensis, Oc. japonicus, Aedes vexans,* and *Culiseta melanura* (11,12). Recent vector competence studies indicate that some North American *Culex* and *Ochlerotatus* species are relatively efficient laboratory vectors (13–15).

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As WNV expands its range westward across North America, examining the vector competence of the different mosquito species will help to anticipate patterns of transmission and the relative contribution of different vector species to virus amplification and persistence. The enzootic transmission cycles of WNV, *Saint Louis encephalitis virus* (SLEV), and W*estern equine encephalomyelitis virus* (WEEV) in North America are conceptually identical, with *Culex* vectors transmitting virus among passerine avian hosts. In the western United States, SLEV and WEEV share a common mosquito host, *Cx. tarsalis*, which will presumably also support WNV transmission. Moreover, WNV and SLEV are closely related viruses in the *Japanese encephalitis virus* (JEV) serocomplex (3), and *Cx. tarsalis* has been shown to be an efficient vector of both SLEV (16) and JEV (17). Current WNV control strategies are based largely on vector control (18); therefore, identifying which species have the greatest potential for transmission is essential in formulating and focusing a prevention plan (19). We evaluated 10 California vector and pest mosquito species' for their ability to become infected with and transmit WNV.

Materials and Methods

Mosquitoes

We assessed the vector competence for WNV of 10 California mosquito species from 14 different geographic locations (Table 1). Vector competence refers to the intrinsic permissiveness of an arthropod for the infection, replication, and transmission of a virus (20,21). Voucher specimens for each species were deposited at the Bohart Museum of Entomology at the University of California, Davis, California. *Cx. tarsalis* is the principal enzootic vector of WEEV and SLEV in California (22). Members of the *Cx. pipiens* complex have been primary vectors of WNV in New York (10) and could potentially play a

Table 1. California mosquito species tested for vector competence for WNV[a]

Species	Source[b]	Generation
Culex tarsalis	Yolo Co.	F_1
	Bakersfield, Kern Co.	F_1
	Coachella Valley, Riverside Co.	F_1
Cx. pipiens quinquefasciatus	Bakersfield, Kern Co.	F_0
	Coachella Valley, Riverside Co.	F_1
	Orange Co.	Wild adults
Cx. p. pipiens	Shasta Co.	F_1
Cx. stigmatosoma	Chino, San Bernardino Co	Wild adults
Cx. erythrothorax	San Joaquin Marsh, Orange Co.	Wild adults
	Coachella Valley, Riverside Co.	Wild adults
Ochlerotatus dorsalis	Morro Bay, San Luis Obispo Co.	F_0
Oc. melanimon	Lost Hills, Kern Co.	Wild adults
Oc. sierrensis	Lake Co.	F_0
Aedes vexans	Coachella Valley, Riverside Co.	F_1
Culiseta inornata	Lost Hills, Kern Co.	F_0

[a]WNV, *West Nile virus*, Co., County; F_0, adults reared from wild-caught larvae or eggs; F_1, progeny from wild-caught adults reared in the laboratory; wild adults, wild-caught adults of unknown age.
[b]All mosquitoes were collected during 2001 except *Cx. p. quinquefasciatus* (Orange Co.), *Cx. stigmatosoma*, *Cx. erythrothorax,* and C*x. inornata*, which were collected during 2002.

similar role in California, especially in urban environments. We defined members of the *Cx. pipiens* complex on the basis of the geographic location of collection and on previously described hybrid zones in California (23). Consequently, we considered members of the complex collected from northern California to be *Cx. p. pipiens*, and those collected from central and southern California to be *Cx. p. quinquefasciatus*. *Cx. stigmatosoma*, an abundant species in California, is naturally infected with WEEV and SLEV (24) and is an efficient laboratory vector of SLEV (16). *Cx. erythrothorax*, another widespread species, typically inhabits marshlands and is an opportunistic feeder (25). *Oc. dorsalis* and *Oc. melanimon*, involved in the transmission of WEEV among small mammals, are laboratory-confirmed vectors of WEEV (26,27). *Oc. sierrensis* is a major pest in California that frequently bites humans and other mammals and transmits dog heartworm, *Dirofilaria immitis* (28). We tested *Ae. vexans* because it feeds readily on mammals (29) and was found to be naturally infected with WNV during the 1999 New York outbreak (11,12). *Cs. inornata* is a mosquito that is active during the winter; this species could potentially maintain WNV amplification and transmission during winter months when *Culex* species are inactive (30).

Virus and Virus Assay

We used WNV strain 35211 AAF 9/23/99, which was isolated from a flamingo during the 1999 New York outbreak and passaged twice in Vero (African green monkey kidney) cell cultures. All artificial blood meal, transmission, and mosquito body samples were examined for virus by plaque assay in six-well tissue culture plates (Costar, Corning, NY) containing monolayers of Vero cells. Mosquito bodies were ground individually in 0.5 mL of mosquito diluent (phosphate-buffered saline [PBS], 20% fetal bovine serum [FBS], antibiotics). Plaque assays were conducted by adding 100 µL of each sample to confluent cell monolayers and incubating inoculated cells at 37°C for 1.5 h to allow for virus to attach and enter cells. After incubation, cells were covered with a 2% agarose overlay containing 0.005% neutral red. After 96 h and 120 h of incubation at 37°C, in a 5% CO_2 atmosphere, plaques were counted, and virus concentrations were calculated as PFUs per 1.0 mL.

Mosquito Infection

Mosquitoes were infected orally by feeding on hanging blood droplets (defibrinated rabbit blood [Microbiological Media, San Ramon, CA]) containing 2.5% sugar and $10^{7.1\pm0.1}$ or $10^{4.9\pm0.1}$ WNV PFUs/1.0 mL of blood. Infectious blood was diluted in bovine albumin-PBS and stored at –80°C until examined by plaque assay to determine the titer. Engorged mosquitoes were held at 28°C, during a 16:8 light:dark photoperiod, and provided a 10% sucrose solution in cotton wicks.

Experimental Transmission

Mosquitoes were deprived of sucrose for 24 h before transmission attempts. On days 7 and 14 after infection, mosquitoes were immobilized by exposure to triethylamine and their proboscises were inserted into a capillary tube containing a 1:1 FBS and 10% sucrose solution for 10 min (31). Transmission fluid was expelled into 250 µL of mosquito diluent and frozen at –80°C until assayed. Individual mosquito bodies were similarly frozen at –80°C before being thawed, ground, and assayed.

Statistical Analysis

Infection and transmission rates were compared at day 7 and day 14 data for each dose by the Fisher exact test using SAS 8.2 (SAS Institute, Inc., Cary, NC). Differences were considered statistically significant at alpha $\geq$0.05 and adjusted for multiple comparisons.

Results

All mosquito species tested were susceptible to infection, and WNV was detected, to some extent, in their salivary secretions. Infection rates were generally higher after 7 days' incubation than 14 days. Transmission rates were generally highest for females infected with the high dose of $10^{7.1\pm0.1}$ PFU/mL and incubated for 14 days (Table 2).

Infection rates varied markedly among species but were consistently highest after infection with the high dose of WNV. Infection rates of *Culex* species and *Cs. inornata* tested 14 days after imbibing the high virus dose ranged from 58% to 100%, except for *Cx. p. quinquefasciatus* from the Coachella Valley and Orange County. *Oc. dorsalis* and *Oc. melanimon*

Table 2. Infection and transmission rates for California mosquito species orally infected with $10^{7.1\pm0.1}$ PFU/mL of *West Nile virus* (WNV)

Species	Source by county	Day transmission attempted	No. tested	Infection rate[a]	Transmission rate[b]
Culex tarsalis	Yolo	7	30	87	60
		14	1	100	100
	Kern	7	15	93	40
		14	35	74	60
	Riverside	7	49	94	10
		14	55	85	62
Cx. pipiens quinquefasciatus	Kern	7	50	86	4
		14	50	58	52
	Riverside	7	60	8	0
		7	60	13	2
		14	58	28	19
	Orange	7	45	80	9
		14	50	66	36
Cx. p. pipiens	Shasta	7	17	100	0
		14	31	100	71
Cx. stigmatosoma	San Bernardino	7	15	67	0
		14	48	77	19
Cx. erythrothorax	Orange	7	15	100	33
		14	25	100	64
Ochlerotatus dorsalis	Kern	7	30	50	13
		14	29	41	34
Oc. melanimon	San Luis Obispo	7	50	46	18
		14	60	48	20
Oc. sierrensis	Lake	7	40	5	3
		14	50	14	6
Aedes vexans	Riverside	14	22	32	23
Culiseta inornata	Kern	14	28	75	21

[a]Percent of mosquito bodies positive for WNV.
[b]Percent of transmission attempts positive for WNV.

infection rates ranged from 41% to 48%. *Ae. vexans* had a moderate infection rate of 32%, whereas *Oc. sierrensis* and *Cx. p. quinquefasciatus* from the Coachella Valley had infection rates <15%. The last two infection rates are significantly lower than the day-14 high–dose infection rates for all species tested, except for *Cx. tarsalis* (Yolo County), *Cx. p. quinquefasciatus* (Orange County), Oc. *dorsalis*, and *Ae. vexans* ($p<0.0009$). Despite the high susceptibility of *Cx. tarsalis* (Yolo County), its day-14 infection rates are not statistically significant, which may be attributed to the small sample size.

Culex species, excluding *Cx. p. quinquefasciatus* from the Coachella Valley and Orange County, were most efficient at transmitting virus after exposure to the high dose and 14-day incubation period. *Cx. tarsalis* (Yolo County) was the most efficient laboratory vector; 60% of expectorate samples contained virus after only 7 days of incubation. These *Cx. tarsalis* (Yolo County) transmission results were significantly higher than all other day-7 high–dose transmission rates ($p<0.001$), except for *Cx. tarsalis* (Bakersfield) and *Cx. erythrothorax* (Coachella Valley). Only one *Cx. tarsalis* (Yolo County) was tested on day 14 because of excessive mortality beginning on day 10. After 14 days of incubation, $\geq$60% of the *Cx. tarsalis* from all three regions in California, *Cx. stigmatosoma* and *Cx. erythrothorax* (Coachella Valley) transmitted virus. *Cx. p. quinquefasciatus* (Bakersfield) followed closely with a 52% transmission rate. *Cx. p. quinquefaciatus* (Orange County), *Cx. p. pipiens, Cx. erythrothorax* (Orange County), *Oc. dorsalis, Oc. melanimon, Ae. vexans*, and *Cs. inornata* had moderate transmission rates ranging from 19% to 36%. *Oc. sierrensis* and *Cx. p. quinquefasciatus* from the Coachella Valley were poor vectors, transmitting virus at rates $\leq$6%. Transmission rates for *Cx. p. quinquefasciatus* (Coachella Valley) were significantly lower than those of *Cx. tarsalis* (Coachella Valley, Bakersfield), *Cx. p. quinquefasciatus* (Bakersfield), *Cx. p. pipiens, Cx. stigmatosoma*, *Cx. erythrothorax* (Coachella Valley), and *Oc. dorsalis* ($p<0.0009$). *Oc. sierrensis* transmission rates were significantly lower than the same six species except for *Oc. dorsalis* ($p<0.0009$).

Table 3. Infection and transmission rates for California mosquito species orally infected with $10^{4.9±0.1}$ PFU/mL of *West Nile virus* (WNV)

Species	Source by county	Day transmission attempted	No. tested	Infection rate[a]	Transmission rate[b]
Culex tarsalis	Yolo	7	25	8	0
		14	11	36	82
	Kern	7	10	30	10
		14	45	7	0
	Riverside	7	40	13	0
		14	10	0	0
Cx. pipiens quinquefasciatus	Kern	7	50	58	0
		14	50	10	0
	Riverside	7	50	0	0
		14	55	0	0
Cx. p. pipiens	Shasta	7	25	36	0
		14	35	23	60
Cx. stigmatosoma	San Bernardino	14	29	69	34
Cx. erythrothorax	Orange	7	47	15	0
	Riverside	7	12	67	0
		14	20	65	30
Ochlerotatus dorsalis	San Luis Obispo	7	29	3	0
		14	25	4	4
Oc. melanimon	Kern	7	50	0	0
		14	60	3	2
Oc. sierrensis	Lake	7	25	4	0
		14	30	0	0

[a]Percent of mosquito bodies positive for WNV.
[b]Percent of transmission attempts positive for WNV.

Infection and transmission rates were lower for mosquitoes exposed to $10^{4.9±0.1}$ PFU/mL of WNV (Table 3) than to the higher dose after both 7 and 14 days of incubation. After 7 and 14 days of incubation, *Culex* species had a wide range of infection rates, except for *Cx. tarsalis* (Coachella Valley) on day 14 and *Cx. p. quinquefasciatus* (Coachella Valley) on days 7 and 14, for which infection was not detectable. *Cx. stigmatosoma* infection rates on day 14 for the low dose were significantly higher than all other day-14 low–dose infection rates, except for *Cx. erythrothorax* (Coachella Valley) and *Cx. tarsalis* (Yolo County) ($p<0.001$). *Cx. erythrothorax* (Coachella Valley) infection rates on day 7 were significantly higher than all day-7 infection rates, except for *Cx. tarsalis* (Bakersfield), *Cx. p. quinquefasciatus* (Bakersfield), and *Cx. p. pipiens* ($p<0.001$). Infection rates for *Cx. erythrothorax* (Coachella Valley) on day 14 also were significantly higher than all day-14 low–dose infection rates except for *Cx. tarsalis* (Yolo County, Coachella Valley), *Cx. p. pipiens*, and *Cx. stigmatosoma* ($p<0.001$). Infection rates for *Ochlerotatus* species were <5% at 7 and 14 days of incubation.

After imbibing a low dose of virus and undergoing 7 days of incubation, positive transmissions were not detected except for *Cx. tarsalis* (Bakersfield). Transmission rates were highest after 14 days of incubation for *Cx. tarsalis* (Yolo County), *Cx. p. pipiens*, *Cx. stigmatosoma,* and *Cx. erythrothorax* (Coachella Valley), although transmission rates for *Cx. tarsalis* (Yolo County) and *Cx. p. pipiens* were higher than the infection rates. Their transmission rates were significantly higher than all others ($p<0.001$). Transmission rates were ≤4% for *Oc. dorsalis* and *Oc. melanimon* after 14 days. WNV transmission was not detected for *Cx. tarsalis* (Coachella Valley), *Cx. p. quinquefasciatus* (Bakersfield and Coachella Valley), *Cx. erythrothorax* (Orange County), and *Oc. sierrensis*. *Ae. vexans* and *Cs. inornata* were not tested at the low dose of virus.

Discussion

All 10 California mosquito species were competent laboratory vectors of WNV, although infection rates varied by species, dose, and incubation period. The amount of virus we used for infection was comparable to published natural WNV avian viremias in Egypt (32) but less than reported for North American birds infected with the NY strain of WNV (33). In addition, artificial blood meals with defibrinated blood may be less infectious by ~2 logs of virus/mL (34), although recent comparisons among *Cx. tarsalis (*infected with WEEV by feeding on viremic chickens or heparinized viremic chicken blood presented by hanging blood droplets, pledgets, or solutions through a biomembrane) did not show significant differences

in infection rates or titers in infected female mosquitoes (F. Mahmood et al., unpub. data). Regardless, all mosquito species became infected and transmitted WNV at some level.

Cx. tarsalis is one of the most efficient laboratory vectors of WNV tested from North America (10,13–15). This species is abundant in California and much of western North America, where it is involved in the maintenance and amplification of WEEV and SLEV (22). Considering its central role in the transmission of arboviruses in avian hosts and its susceptibility to WNV infection in the laboratory, *Cx. tarsalis* has the greatest potential of the species we studied to amplify and maintain WNV in California.

Mosquitoes in the *Cx. pipiens* species complex also may be an important enzootic mosquito host in California. *Cx. p. pipiens* was identified as a primary WNV vector during the 1999 New York outbreak (10) and has been suggested as a host for overwintering flaviviruses such as WNV and SLEV (35–38). This species could play a similar role in WNV transmission in California. *Cx. p. pipiens* is mainly ornithophilic (39), but *Cx. p. quinquefasciatus* feeds readily on mammals (25,40), potentially transferring WNV from birds to humans and horses.

Cx. p. quinquefasciatus from Coachella Valley and Orange County were significantly less susceptible to infection than those collected from Bakersfield in the southern Central Valley. Differences in infection and transmission rates indicated that geographic differences may exist in the vector competence for WNV of mosquitoes within this species complex, which could relate to the introgression of *Cx. p. pipiens* genes into the Bakersfield population (23). The extent to which differences in infection and transmission are caused by the genetic structure of mosquito populations throughout the state and the impact of these differences on WNV transmission require additional study.

Results for *Cx. tarsalis* (Yolo County) and *Cx. p. pipiens* exposed to the low dose of virus and incubated for 14 days were unexpected. Infection rates for both species were consistent with results for most *Culex* species, but transmission rates were high and exceeded infection rates (i.e., some positive expectorate samples were not associated with positive results for the associated mosquito bodies, even after retesting). These incongruous results may be attributed to experimenter error. Additional replicates of these experiments may be needed to verify our results.

Infection with WNV may have increased death rates in infected female mosquitoes. In most groups, infection rates after 14 days were less than infection rates after 7 days, perhaps indicating that susceptible females died more rapidly than less susceptible or uninfected females. Most noticeable were the synchronous deaths of *Cx. tarsalis* in both the high- and low-dose groups from the highly susceptible Yolo County population after 10 days of incubation.

Cx. stigmatosoma and *Cx. erythrothorax* are widely distributed species in California and were highly susceptible to WNV infection. *Cx. stigmatosoma* preferentially feeds on birds and may play a role as an enzootic vector. Conversely, *Cx. erythrothorax* behaves as an opportunistic feeder, potentially bridging WNV transmission between birds and mammals (25).

In California, *Oc. dorsalis* and *Oc. melanimon* are involved in the transmission of WEEV among small mammals and are both laboratory-confirmed vectors of WEEV (26,27). Both species have a similar ecology and can be found in fresh water; however, *Oc. dorsalis* also develops in saline and alkaline habitats in coastal and southeastern California, respectively (26,41,42). *Oc. melanimon* plays a secondary role in the maintenance of WEEV in lagomorphs during the late summer in the Central Valley of California (43). WEEV and California encephalitis viruses have been isolated from *Oc. melanimon* (44,45). *Oc. melanimon* is an abundant pest species in the Central Valley that readily bites humans, other mammals, and (occasionally) birds (29,46). With moderate WNV transmission rates and a preference for mammalian hosts, these species have little potential to act as secondary or bridge vectors from birds to mammals.

Oc. sierrensis, a widely distributed tree hole mosquito, is a major pest in California that frequently bites humans and other mammals (28,46). However, arboviruses have not been isolated from this species to date, and its infection and transmission rates for WNV were low in the current study. Mammalian feeding preferences coupled with low vector competence for WNV indicate that this species probably would not be an enzootic or bridge vector of WNV in California.

WNV was isolated from wild *Ae. vexans* collected from the eastern United States during 2001 (11). Arboviruses rarely have been isolated from *Ae. vexans* in California (24), even though this species has been found infected with WEEV during epizootics in the central United States (47) and has been shown capable of laboratory transmission of WEEV (48) and SLEV (49) at high infectious doses. In a single trial during the current study, *Ae. vexans* exhibited moderate infection and transmission rates for WNV. Mammalian feeding preferences (29,45) decrease its potential as an enzootic vector for WNV in California.

Cs. inornata is a widely distributed winter mosquito in California with relatively high infection and moderate transmission rates for WNV. The species is a laboratory-confirmed vector of WEEV and SLEV viruses (49,50) and a primary horizontal and vertical vector of some bunyaviruses (51,52). We tested this species because of its potential to extend the transmission season of WNV in California beyond the November–January diapause of *Cx. tarsalis* (53,54). *Cs. inornata* primarily feeds on livestock and occasionally on birds (46,55,56) and may play a minor role in the amplification and transmission of WNV in California.

Because WNV was recently introduced into North America, little is known about the vector competence of New World mosquitoes for this invading strain of virus. Assessing the vector competence of California mosquitoes provides arbovirus surveillance and mosquito control programs with valuable

information concerning the possible roles of different species in the transmission and maintenance of WNV. Our results indicated that, similar to other parts of the world, mosquitoes in the genus *Culex* are anticipated to be the principal enzootic mosquito hosts of WNV in California. On the basis of their vector competence and host-feeding patterns, *Cx. tarsalis* may be the principal vector in rural agricultural ecosystems; in addition, members of the *Cx. pipiens* complex and perhaps *Cx. stigmatosoma* will be important vectors in urban settings. If WNV becomes established in a *Cx. tarsalis*–passerine transmission cycle, the effect of sharing a common vector on the evolution of two closely related flaviviruses, WNV and SLEV, will be determined. The variation in WNV vector competence and other components of vectorial capacity within single mosquito species will need to be studied. *Cx. erythrothorax* and species in the genera *Ochlerotatus* and *Culiseta* are likely to serve as secondary or bridge vectors. Our results for *Cx. p. quinquefasciatus* collected in different geographic locations, however, indicate that not all mosquitoes in a single taxonomic unit will contribute equally to WNV transmission.

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Ms. Goddard is a graduate student in the Entomology Department at the University of California, Davis. Her research focuses on the evolution of w*estern equine encephalomyelitis virus* (WEEV) as it is serially passaged in different insect vectors and the correlation between phenotypic and genotypic changes in WEEV as it replicates in different vector species.

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Efficacy of Killed Virus Vaccine, Live Attenuated Chimeric Virus Vaccine, and Passive Immunization for Prevention of *West Nile virus* Encephalitis in Hamster Model

Robert B. Tesh,* Juan Arroyo,† Amelia P.A. Travassos da Rosa,* Hilda Guzman,* Shu-Yuan Xiao,* and Thomas P. Monath†

Results of experiments evaluating the efficacy of three immunization strategies for the prevention of *West Nile virus* (WNV) encephalitis are reported. Immunization strategies evaluated included a killed virus veterinary vaccine, a live attenuated chimeric virus vaccine candidate, and passive immunization with WNV-immune serum; all were tested by using a hamster model of the disease. Each product protected the animals from clinical illness and death when challenged with a hamster-virulent wild-type WNV strain 1 month after initial immunization. The live attenuated chimeric virus vaccine candidate induced the highest humoral antibody responses, as measured by hemagglutination inhibition, complement fixation, and plaque reduction neutralization tests. Although the duration of protective immunity was not determined in this study, our preliminary results and the cumulative experience of other virus vaccines suggest that the live attenuated chimeric virus provides the longest lasting immunity.

After the appearance of *West Nile virus* (WNV) in North America and the resulting human and equine cases of encephalitis, considerable efforts have focused on developing vaccines against this emerging viral pathogen. A number of different WNV vaccine candidates have been recently described and are now in various stages of testing (1–4). A formalin-inactivated veterinary vaccine (West Nile Virus Vaccine, Killed, Fort Dodge Animal Health, Fort Dodge, IA) was conditionally licensed by the U.S. Department of Agriculture in August 2001 and has already been used in equines and exotic zoo birds in some areas of the country. We report the results of studies evaluating the efficacy of the killed veterinary vaccine, a live attenuated chimeric virus candidate, and passive immunization with immune serum for preventing WNV encephalitis in a hamster model of the disease (5,6).

Materials and Methods

Virus, Vaccines, and Immune Serum

The virus used to infect animals in these studies was a second Vero cell passage of strain NY385-99, originally isolated from the liver of a Snowy Owl (*Nyctea scandiaca*) that died at the Bronx Zoo during the 1999 WNV epizootic in New York City (7). Two different WNV vaccines were evaluated in the hamster model: West Nile Encephalitis Virus Vaccine (killed), (Fort Dodge Animal Health, Fort Dodge, IA), a formalin-inactivated whole virus veterinary vaccine, and ChimeriVax–West Nile virus (Acambis, Inc., Cambridge, MA), a live attenuated chimeric virus vaccine candidate (1). The immune serum used in passive immunization experiments was prepared by pooling convalescent-phase serum samples of six hamsters that were bled 5 weeks after infection with WNV strain NY385-99.

Hamsters and Hamster Model

Animals used in these studies were adult (10–11 weeks old) female hamsters (*Mesocricetus auratus*) obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The hamster model of WNV encephalitis has been described (5,6). After intraperitoneal inoculation of 10^4 tissue culture infectious $dose_{50}$ ($TCID_{50}$) of WNV strain NY385-99, fatal encephalitis developed in approximately 50% of adult hamsters. Animals were cared for in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council) under an animal-care protocol approved by the University of Texas Medical Branch. All work with infected animals was carried out in biosafety level 3 facilities.

Immunization Schedule and Challenge with WNV

Hamsters immunized with West Nile Encephalitis Virus Vaccine (killed) received two intramuscular injections of 0.1 mL

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each, given 3 weeks apart, following manufacturer's recommendation. Eleven days after the second immunization, the hamsters were bled to determine their antibody response; each animal was then inoculated intraperitoneally with 10^4 $TCID_{50}$ of WNV strain NY385-99. After challenge with live virus, the animals were bled daily for 6 consecutive days to measure the level of viremia and subsequent immune response. The animals were observed for another 21 days to determine if any developed signs of encephalitis or died.

Two groups of hamsters were inoculated intramuscularly with the ChimeriVax–WNV vaccine. One group received $10^{6.3}$ PFU, and the other received $10^{3.3}$ PFU of the chimeric virus. Thirty-one and 32 days, respectively, after injection of the virus, the two groups of hamsters were inoculated intraperitoneally with 10^4 $TCID_{50}$ of WNV strain NY385-99. After challenge, the animals were bled daily for 6–7 days to measure the level of viremia and immune response, as described previously. These hamsters were also observed for 21 additional days for signs of illness or death.

Two groups of hamsters also were passively immunized with different amounts (0.1 mL and 0.5 mL) of hamster WNV-immune serum, given intramuscularly. One day after passive immunization, both groups of animals were bled to determine if detectable titers of WNV hemagglutination inhibition (HI) antibodies were present in their serum. The animals then were inoculated intraperitoneally with 10^4 $TCID_{50}$ of WNV strain NY385-99, bled for 7 consecutive days, and observed for another 21 days, as described previously.

A group of naïve (control) hamsters was also inoculated intraperitoneally with the same dosage of WNV strain NY385-99. Thirty-eight days after infection, 10 of the surviving animals were bled to determine their antibody response.

Virus Titration and Antibody Determinations

Serial blood samples from the hamsters were titrated in microplate cultures of the C6/36 clone of *Aedes albopictus* cells (8). The presence or absence of WNV viral antigen, determined by immunofluorescence, was used as the endpoint. This technique has been described in detail (5,6). WNV titers in the blood samples were calculated as the $TCID_{50}$ per microliter of specimen by the method of Reed and Muench (9).

Serum antibodies to WNV and *Yellow fever virus* (YFV) were measured by HI, complement fixation (CF), and plaque reduction neutralization (PRN) tests. Antigens for HI and CF tests were prepared from brains of newborn mice injected intracerebrally with the respective flaviviruses; these infected brains were treated by the sucrose-acetone extraction method (10). Hamster sera were tested by HI at serial twofold dilutions from 1:20 to 1:5,120 at pH 6.6 (WNV) or 6.4 (YFV) with 4 U of antigen and a 1:200 dilution of goose erythrocytes, following established protocols (10).

CF tests were performed by a microtechnique (10) with two full units of guinea pig complement and antigen titers $\geq$1:32. Titers were recorded as the highest dilutions giving +3 or +4 fixation of complement on a scale of 0 to +4.

PRN tests on hamster serum were performed by a previously described technique (11) in 24-well, Vero-microplate-cell cultures, using a fixed virus inoculum (~100 PFU) against varying serum dilutions (1:10 to 1:20,480). For PRN tests, the Egypt 101 strain of WNV (12) was used because this strain produced larger and sharper plaques than NY385-99. Hamster serum samples were diluted in phosphate-buffered saline, pH 7.4, containing 10% fresh guinea pig serum. Virus inoculum was mixed with an equal volume of each serum dilution; and the mixture was incubated overnight at 4°C. The following day, 50 uL of the serum-virus mixture was injected into Vero microplate cultures, with two wells per serum dilution. Virus plaques were read 4 days later; $\geq$90% plaque reduction was used as the endpoint.

Results

WNV Infection in Naïve Hamsters

The level and duration of viremia, antibody response, and deaths in naïve (non-immune) adult hamsters after WNV infection have been described (5,6). Following intraperitoneal inoculation of 10^4 $TCID_{50}$ of WNV strain NY385-99, moderate levels of viremia that persisted for 6 days developed in the hamsters (Figure). HI antibodies were detected in the animals as early as day 5, and titers continued to increase through day 7. Initially, HI antibody response in primary WNV infection is specific; but after 3 or 4 weeks, the antibody pattern becomes more broadly reactive and serologic cross-reactions occur with other flavivirus antigens (6). Table 1 shows the HI, CF, and PRN antibody responses to WNV antigen and virus in 10 naïve adult hamsters that survived infection with the NY385-99 virus strain. These animals were bled 38 days after infection. Hamsters who survived infection with wild-type WNV appeared to have solid immunity 1 month after infection (Table 1). Convalescent-phase sera from some of these animals were used to prepare the WNV immune serum used in the passive immunization experiments described below.

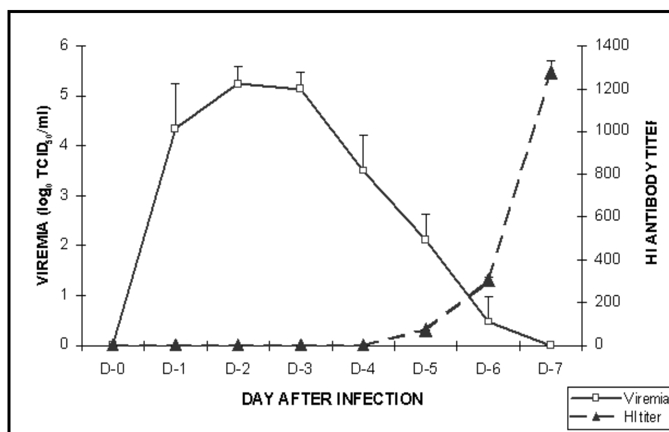

Figure. Daily mean (plus or minus the standard deviation) virus titers and hemagglutination inhibition (HI) antibody levels in 10 naïve (control) hamsters after intraperitoneal inoculation of 10^4 $TCID_{50}$ *West Nile virus* strain NY385-99.

Table 1. Hemagglutination inhibition, complement fixation, and plaque reduction neutralization antibody responses of naïve adult golden hamsters that survived *West Nile virus* infection[a,b]

Animal no.	HI antibody titer WNV antigen	CF antibody titer WNV antigen[c]	WNV PRN antibody titer[d]
H-8589	1:1280	1:320	1:10,240
H-8590	1:640	1:160	1:10,240
H-8591	1:640	1:320	1:5120
H-8592	1:640	1:80	1:2560
H-8593	1:640	1:160	1:2560
H-8594	1:1280	1:160	1:5120
H-8595	1:1280	1:320	1:10,240
H-8596	1:1280	1:320	≥1:20,480
H-8597	1:1280	1:320	1:5120
H-8598	1:1280	1:640	1:2560

[a]HI, hemagglutination inhibition; CF, complement fixation; PRN, plaque reduction neutralization; WNV, *West Nile virus*.
[b]Animals 38 days after inoculation of virus strain NY385-99 ($10^{4.0}$ tissue culture infective $dose_{50}$ given intraperitoneally).
[c]WNV antigen titer = ≥1:32.
[d]Highest serum dilution producing >90% plaque inhibition.

Table 2. WNV antibody response of hamsters after immunization with WNV killed virus vaccine (Fort Dodge) and challenge with the NY385-99 strain of WNV[a,b]

Animal no.	HI antibody titer	CF antibody titer	PRN antibody titer[c]
32 days after Ft. Dodge vaccine			
H-8440	1:40	1:40	1:20
H-8441	0[d]	0	<1:10
H-8442	1:20	1:40	<1:10
H-8443	1:40	1:40	1:20
H-8445	1:40	1:40	1:20
H-8446	1:20	1:20	1:40
H-8447	1:20	1:20	<1:10
H-8448	1:20	1:20	<1:10
H-8449	1:40	1:20	1:10
6 days after challenge with WNV			
H-8440	1:80	1:80	ND
H-8441	1:320	1:160	ND
H-8442	1:40	1:40	ND
H-8443	1:40	1:40	ND
H-8445	1:320	1:160	ND
H-8446	1:80	1:40	ND
H-8447	1:320	1:160	ND
H-8448	1:160	1:80	ND
H-8449	1:160	1:40	ND

[a]WNV, *West Nile virus*; HI, hemagglutination inhibition; CF, complement fixation; PRN, plaque reduction neutralization; ND, not done.
[b]$10^{4.0}$ 50% tissue culture infective $dose_{50}$ given intraperitoneally.
[c]Highest serum dilution producing >90% plaque inhibition.
[d]0 = <1:20.

WNV Infection in Hamsters Previously Immunized with a Killed Vaccine

Table 2 shows the HI, CF, and PRN antibody responses of nine hamsters 32 days after immunization (2 injections) with the Fort Dodge WNV killed vaccine. One month after the initial immunization, eight of nine animals had detectable levels of HI and CF antibodies; five of nine hamsters had low levels of WNV-neutralizing antibodies. Six days after challenge with the wild-type virus, most of the hamsters had an increase (range two-fold to 32-fold increase) in their HI and CF antibody titers, indicating some degree of antigenic stimulation and possible virus replication. Two of the hamsters had detectable levels of viremia after challenge with the wild-type virus (Table 3). However, none of the animals appeared clinically ill, and all survived.

WNV Infection in Hamsters Previously Immunized with Live, Attenuated Chimeric Vaccine

Tables 4 and 5 show the antibody responses of hamsters receiving two different doses ($10^{6.3}$ and $10^{3.3}$) of ChimeriVax-WNV vaccine. The results of these two experiments were similar. One month after immunization, all animals had detectable HI, CF, and PRN-antibody titers to WNV. When tested 6–7 days after challenge with the wild-type virus, none of the animals had a substantial change in antibody titer. WNV was detected in the blood of one animal on day 2; the titer was $10^{0.7}$ $TCID_{50}$/mL (data not shown). None of the animals in these two groups appeared sick, and all survived.

WNV Infection in Passively Immunized Hamsters

Two groups of hamsters (A and B) were inoculated with 0.5 mL and 0.1 mL, respectively, of hamster WNV-immune serum (Table 6). HI antibody titer of the immune serum was 1:1,280; PRN titer was 1:5,120. Twenty-four hours later, the animals were bled and then injected with the wild-type virus. When tested 24 hours after receiving WNV-immune serum, all

Table 3. Level and duration of viremia in hamsters previously immunized with WNV killed virus vaccine (Fort Dodge) and challenged with the NY385-99 strain of WNV[a]

Animal no.	Postinfection, by day					
	1	2	3	4	5	6
8440	0[b]	0	0	0	0	0
8441	0	1.2	3.5	1.3	1.2	0
8442	0	0	0	0	0	0
8443	0	0	0	0	0	0
9445	0	0	0	0	0	0
8446	0	0	0	0	0	0
8447	0	0	0	0	0	0
8448	0	0.7	3.0	4.3	2.3	0
8449	0	0	0	0	0	0

[a]WNV, *West Nile virus*.
[b]Virus titers in blood expressed as log_{10} 50% tissue culture infective $dose_{50}$/mL of blood. 0 = <$10^{0.7}$ $TCID_{50}$.

Table 4. Antibody response of adult golden hamsters to intramuscular inoculation of 1.8 X 10^6 of ChimeriVax–WNV and subsequent challenge with WNV ($10^{4.0}$ $TCID_{50}$ given intraperitoneally)[a]

	HI antibody titer		CF antibody titer		WNV PRN antibody titer[b]
Animal no.	YFV antigen	WNV antigen	YFV antigen	WNV antigen	
31 days after ChimeriVax-WNV					
H-8183	1:40	1:160	0[c]	0	1:160
H-8184	1:40	1:160	0	ND	1:160
H-8185	1:40	1:160	0	1:20	1:640
H-8186	1:80	1:320	0	1:40	1:320
H-8187	1:40	1:160	0	1:20	≥1:640
H-8188	1:40	1:160	0	1:20	1:640
H-8189	1:80	1:320	0	1:40	1:640
H-8190	1:40	1:160	0	1:20	1:320
H-8191	1:80	1:160	0	1:20	1:80
H-8192	1:40	1:160	0	1:40	1:80
6 days after challenge with WNV					
H-8183	1:20	1:160	0	0	ND
H-8184	1:40	1:160	0	1:40	ND
H-8185	1:20	1:160	0	1:20	ND
H-8186	1:80	1:320	0	1:40	ND
H-8187	1:20	1:160	0	1:20	ND
H-8188	1:40	1:160	0	1:20	ND
H-8189	1:80	1:320	0	1:40	ND
H-8190	1:40	1:320	0	1:40	ND
H-8191	1:40	1:320	0	1:20	ND
H-8192	1:40	1:160	0	1:20	ND

[a]WNV, *West Nile virus*; HI, hemagglutination inhibition; CF, complement fixation; PRN, plaque reduction neutralization; YFV, *Yellow fever virus*; ND, not done.
[b]Highest serum dilution producing ≥90% plaque inhibition.
[c]0 = <1:20.

animals in group A had low but detectable HI antibody titers. No HI antibodies in group B were detectable after 24 hours. Seven days after challenge with WNV, one animal in group A still had detectable HI antibodies. Hamsters in groups A and B were bled for 6 consecutive days; no virus was detectable in any of the blood samples (data not shown). All animals in groups A and B appeared well and survived challenge. The absence of an antibody response or viremia in the passively immunized animals suggests that no virus replication occurred after challenge with WNV.

Discussion

Each of the three immunization products evaluated in this study (killed whole virus vaccine, live attenuated chimeric virus vaccine, and passive immunization with immune serum) protected hamsters from clinical encephalitis and death upon subsequent challenge with the virulent wild-type WNV strain NY385-99. In contrast, fatal encephalitis developed in approximately 50% of naïve hamsters inoculated with the same virus dose (5). One obvious deficiency of our study was that the duration of protection induced by each immunization product was not determined. Determining the duration of protection is difficult with a relatively short-lived (approximately 2 yrs) animal such as a hamster; such studies are more meaningful when conducted by using longer-lived species such as horses or humans. Nonetheless, the general experience with other live and inactivated vaccines and the use of immune globulin for prevention of viral diseases offers some clue as to the advantages and disadvantages of the three approaches for preventing WNV encephalitis (13,14).

Passive Immunization with WNV Immune Globulin

Hamsters inoculated with WNV immune serum (0.5 mL and 0.1 mL) appeared to be completely protected when challenged with the wild-type virus 24 hours after passive immunization (Table 6). No virus was detected in the animals' blood after challenge, and HI antibodies to WNV viral antigen did not develop. Immune globulins present in the WNV-immune serum probably inhibited virus replication; consequently, insufficient antigenic mass existed in the animals to stimulate an antibody response. One advantage of passive immunization with WNV-immune globulins is that the protective effect is almost immediate (<24 hours in the case of the hamsters in our experiment). Passive immunization with WNV-immune glob-

Table 5. Antibody response of adult golden hamsters to intramuscular inoculation of 1.8 X 10^3 of ChimeriVax-WNV and WNV$10^{4.0}$ $TCID_{50}$ given intraperitoneally[a]

Animal no.	HI antibody titer		CF antibody titer		WNV PRN antibody titer[b]
	YFV antigen	WNV antigen	YFV antigen	WNV antigen	
32 days after ChimeriVax-WNV					
H-8322	1:40	1:160	0[c]	1:20	1:320
H-8323	1:20	1:80	0	1:40	1:160
H-8324	1:80	1:320	1:20	1:80	1:160
H-8325	1:40	1:320	0	1:80	≥ 1:640
H-8326	1:40	1:320	0	1:80	1:320
H-8327	1:40	1:320	ND	ND	1:320
H-8328	1:80	1:320	1:20	1:80	1:80
H-8329	1:40	1:320	0	1:80	1:80
7 days after challenge with WNV					
H-8322	1:40	1:320	0	1:20	ND
H-8323	1:40	1:160	0	1:20	ND
H-8324	1:80	1:320	0	1:40	ND
H-8325	1:80	1:320	0	1:80	ND
H-8326	1:80	1:320	0	1:40	ND
H-8327	1:40	1:160	0	1:20	ND
H-8328	1:80	1:320	0	1:40	ND
H-8329	1:40	1:320	0	1:80	ND

[a]WNV, *West Nile virus*; HI, hemagglutination inhibition; CF, complement fixation; PRN, plaque reduction neutralization; YFV, *Yellow fever virus*; ND, not done.
[b]Highest serum dilution producing ≥90% plaque inhibition.
[c]0 = <1:20.

ulins might be desirable when rapid, temporary protection against the virus is needed or when a person with a compromised immune system requires protection. The major disadvantage of passive immunity acquired from immune globulins is the relatively short period of protection (13).

Inactivated WNV Vaccine

Most hamsters immunized with the Fort Dodge killed WNV vaccine had low levels of HI, CF, and PRN antibodies after two injections (Table 2). None of the animals that received the killed vaccine appeared clinically ill or died after challenge with the wild-type WNV. However, six of the nine hamsters had a substantial increase in their HI antibody titer after challenge with the wild-type virus; two of the nine animals subsequently had detectable viremia (Table 3). These data suggest that the immune response to the killed vaccine was insufficient to completely inhibit virus replication and that some degree of virus replication occurred after challenge with the wild-type virus.

The Fort Dodge WNV veterinary vaccine used in this study is a commercially available formalin-inactivated whole virion preparation that has received conditional approval from the U.S. Department of Agriculture for use in horses. The first WNV vaccine approved for use in the United States, its substrate and degree of purification are not public information. Duration of protection with this vaccine is also unknown, although the manufacturer recommends that horses be immunized annually.

The major advantage of killed vaccines is their safety; the disadvantages are that they often require multiple doses to elicit and sustain an effective immune response and that the immune response may be imbalanced, leading to subsequent potentiation of the disease (14,15). However, highly purified killed-virus vaccines have been used effectively in persons for the prevention of *Japanese encephalitis virus* and *Tick-borne encephalitis virus* (16,17).

Live, Attenuated Chimeric WNV

Hamsters receiving both doses ($10^{6.3}$ or $10^{3.3}$ PFU) of the ChimeriVax-WNV had good HI and PRN-antibody responses to WNV, when tested 1 month after immunization (Tables 4 and 5). One animal (H-8183) did not develop CF antibodies after immunization, and two other hamsters were not tested. Notably, CF antibodies to WNV viral antigen developed in most of the hamsters after vaccination with the ChimeriVax-WNV, although humans without previous flavivirus exposure generally do not develop CF antibodies after administration of the 17D vaccine (18). The ChimeriVax technology platform uses YFV 17D as a live vector for envelope genes of WNV (1).

Six and 7 days after challenge with the wild-type virus, we found no change in HI or CF titers of the animals previously immunized with the ChimeriVax-WNV (Tables 4 and 5). PRN titers were not tested after challenge. WNV was detected in the blood of a single animal on day 2, although WNV antibody titers did not increase in the blood of this hamster 6 days after

Table 6. WNV hemagglutination inhibition antibody titers in adult hamsters 24 h after inoculation (passive immunization) with WNV immune serum, and 7 days later after challenge with WNV ($10^{4.0}$ $TCID_{50}$ given intraperitoneally)[a]

Hamster no.	24 h after passive immunization	7 days after challenge with WNV
Group A – received 0.5 mL WNV immune serum		
H-8126	1:40[b]	0
H-8127	1:20	0
H-8128	1:20	0
H-8129	1:20	0
H-8130	1:20	0
H-8138	1:40	0
H-8139	1:40	1:10
Group B – received 0.1 mL WNV immune serum)		
H-8131	0	0
H-8132	0	0
H-8133	0	0
H-8134	0	0
H-8135	0	0
H-8136	0	0
H-8137	0	0

[a]WNV, *West Nile virus*.
[b]Hemagglutination inhibition antibody titer. 0 = <1:10.

challenge. These data suggest that minimal WNV replication occurred in the animals immunized with the chimeric virus when challenged with the wild-type virus. The levels of PRN antibodies present in ChimeriVax-WNV–immunized hamsters also suggest that the protection would be long lasting.

The major advantages of live attenuated virus vaccines are that they induce a more balanced immune response and that the resulting immunity is longer lasting than with killed vaccines or immune globulins (13–15). Major concerns with a live WNV vaccine are related to safety: 1) a potential vaccine might contain adventitious agents; 2) the vaccine virus might cause illness in some recipients or lose attenuation during manufacture or replication; and 3) stability. The second and third concerns are currently being investigated and addressed; results will be reported in subsequent publications. However, on balance, the ChimeriVax-WNV candidate vaccine appears to be quite effective in preventing WNV encephalitis, on the basis of our comparative studies in a hamster model of the disease.

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RESEARCH

Mass Vaccination Campaign Following Community Outbreak of Meningococcal Disease

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During December 12–29, 1998, seven patients ages 2–18 years were diagnosed with serogroup C meningococcal disease in two neighboring Florida towns with 33,000 residents. We evaluated a mass vaccination campaign implemented to control the outbreak. We maintained vaccination logs and recorded the resources used in the campaign that targeted 2- to 22-year-old residents of the two towns. A total of 13,148 persons received the vaccinations in 3 days. Vaccination coverage in the target population was estimated to be 86% to 99%. Five additional cases of serogroup C meningococcal disease occurred in the community during the year after the campaign began, four in patients who had not received the vaccine. The cost of control efforts was approximately $370,000. Although cases continued to occur, the vaccination campaign appeared to control the outbreak. Rapid implementation, a targeted approach, and high coverage were important to the campaign's success.

Neisseria meningitidis is a leading cause of bacterial meningitis and sepsis in children and young adults in the United States (1,2). An estimated 2,600 cases occur each year, most of them sporadic (2). Between 10 and 15 outbreaks of meningococcal disease are reported in the country annually (1,3). Outbreaks can occur in institutions as well as in communities. Communitywide outbreaks can persist for several months, and controlling them remains a major challenge in public health (4–6).

The primary method for preventing sporadic meningococcal disease is chemoprophylaxis of close contacts after a case is identified (1,7,8). However, the protective effect of chemoprophylaxis is of limited duration (6,9,10).

A quadrivalent polysaccharide meningococcal vaccine effective against *N. meningitidis* serogroup A, C, W135, and Y is available in the United States (7,11). Serogroup C *N. meningitidis* accounts for most U.S. outbreaks (3). The Advisory Committee on Immunization Practices (ACIP) has released recommendations for the use of meningococcal vaccine to control outbreaks of serogroup C meningococcal disease (11).

Identifying the need for a vaccination campaign, defining the target population, implementing the campaign rapidly, and achieving high vaccination coverage are difficult (5). Mass vaccination campaigns require major logistic efforts and often take place in an atmosphere of public anxiety (5,6,12,13). Few local and state health departments have much experience in responding to such outbreaks. Mass vaccination campaigns in response to meningococcal disease outbreaks have been reported before, but only limited information is available on the operational aspects of such efforts (4,5,9,14).

In December 1998, two neighboring towns with a combined population of 33,000 persons in Putnam County, Florida, had a community outbreak of meningococcal disease (1,3). The health department administered chemoprophylaxis to close contacts of the case-patients and investigated links between patients. Detailed results of the investigation have been described elsewhere (15). On December 29, 1998, the decision was made to implement a mass vaccination campaign to control the outbreak in the community, based on ACIP recommendations (7). We describe the epidemiology of the outbreak and the methods used for providing chemoprophylaxis and implementing the vaccination campaign. We also evaluate the results and the cost of the control efforts.

Materials and Methods

Case Definition

A case-patient was defined as a person in Putnam County with onset of clinically compatible illness after November 1998 and isolation of serogroup C *N. meningitidis* obtained from a normally sterile site or detection of serogroup C meningococcal polysaccharide antigen in the cerebrospinal fluid or serum (7). Pulsed-field gel electrophoresis (PFGE) was performed on culture-confirmed isolates in the state health department laboratory by using previously described methods (16,17).

Chemoprophylaxis

Chemoprophylaxis—rifampin, ciprofloxacin, or ceftriaxone, as recommended by ACIP (7)—was given to close contacts. A close contact was defined as a household member,

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day-care center contact, or anyone directly exposed to the patient's oral secretion (7,18). We documented all chemoprophylaxis provided by the health department for the first nine patients and retrospectively verified whether the persons who had received prophylaxis met the definition of a close contact.

Vaccination Campaign

The decision to implement the vaccination campaign was based on ACIP guidelines for community-based outbreaks (7). We defined the population at risk as the population of the two neighboring towns since this area represented the smallest geographically contiguous population that included all case-patients. The target population for the vaccination campaign was defined as all residents ages 2–22 years.

The vaccination campaign took place December 30–31, 1998, and January 2, 1999. Additional vaccinations were offered at the health department by appointment until January 20, 1999, for those who belonged to the target group but did not receive the vaccine during the 3-day campaign.

We organized a single-site immunization clinic in the high school cafeteria. We used Menommune quadrivalent polysaccharide meningococcal vaccine (Aventis Pasteur [formerly Pasteur Merieux Connaught], Swiftwater, PA) in 50-dose vials originally designed for jet injector use. All adult vaccinees signed a consent form before being vaccinated. A parental consent was required for vaccinees <18 years of age.

Public Information

Staff of the county health department, the state health department, and the school administration received question-and-answer sheets to respond to telephone inquiries from the public. The state health department installed a toll-free phone number with automated information on the vaccination campaign and posted additional information on the Internet. Health department staff held daily press conferences from December 30, 1998, through January 2, 1999.

Vaccination Coverage

We maintained vaccination logs during the campaign, in which we documented age, sex, and address of the vaccinee as well as date of vaccination. To determine the reliability of the address information in the log, we selected a random sample from the vaccinees listed in the log. We validated the address information with the school enrollment database of the county. Only addresses of persons identified in both databases were compared. The size of the population at risk and the target population was determined by using 1998 official Florida population figures provided by the Bureau of Economic and Business Research at the University of Florida.

Cost of the Intervention

We recorded expenditures for health department staff time and material resources associated with the efforts to control the outbreak. We did not include salaries for police and fire department personnel, local health-care providers, or the value of time and services donated by volunteers.

Results

Outbreak Investigation

During December 14–26, 1998, four female and three male case-patients of serogroup C meningococcal disease were reported to the health department of Putnam County (Table 1). Their ages ranged from 2 to 18 years (median 12). Two of the patients had close contact with each other so that the primary disease attack rate was 15 per 100,000. The county had received an average of one report of meningococcal disease per year in 1988–1998.

Table 1. Cases of serogroup C meningococcal disease, Putnam County, Florida, 1998–1999[a]

Onset date	Sex	Age	Resident of target area	Received chemoprophylaxis	Received vaccine	PFGE	Outcome
12/12/98	F	2 yrs	Yes	No	No	No isolate	No sequelae
12/12/98	F	18 yrs	Yes	No	No	Identical	No sequelae
12/18/98	M	12 yrs	Yes	No	No	Identical	No sequelae
12/25/98	F	2 yrs	Yes	No	No	Identical	No sequelae
12/25/98	F	4 yrs	Yes	No	No	Identical	No sequelae
12/26/98	M	11 yrs	Yes	No	No	Identical	No sequelae
12/26/98	M	12 yrs	Yes	No	No	No isolate	No sequelae
12/29/98	M	18 yrs	Yes	No	No	Identical	Died
01/03/99	M	25 yrs	Yes	Yes	No	Identical	No sequelae
3/25/99	M	17 yrs	No	No	No	Identical	No sequelae
5/12/99	F	6 yrs	Yes	Yes	Yes	3 bands different	Necrosis of ear lobe
12/28/99	F	3 mo	Yes	No	No	Identical	Died

[a]PFGE, pulsed-field gel electrophoresis; M, male; F, female.

One case occurred the night before the campaign started. After the campaign began, four additional cases (two female, two male) of serogroup C meningococcal disease occurred until December 1999; ages of patients ranged from 3 months to 25 years (Table 1). One of the patients with onset in January 1999 had received chemoprophylaxis. Another patient, whose onset was in May, had received both chemoprophylaxis and vaccine. The other postcampaign patients had not received vaccine. PFGE performed on isolates from 10 of 12 patients showed identical patterns for the first eight isolates. The isolates from the three case-patients in March, May, and December 1999 differed by three or fewer bands from the earlier outbreak strains (Table 1).

Chemoprophylaxis

The health department gave chemoprophylaxis to a total of 484 contacts, ranging from 7 to 108 contacts per patient. Three hundred six (63%) of those who received chemoprophylaxis were considered close contacts according to ACIP criteria (7).

Vaccination Campaign

Law enforcement personnel from the local sheriff's department provided crowd and traffic control. Volunteers and clerical staff served as welcome staff, handed out information sheets, and guided incoming people to the lines. The main waiting line outside the school was divided into 6–18 parallel lines inside the cafeteria. One check-in station existed for each line. Each such station was staffed with one to three clerks, who determined eligibility for persons by age and residency requirements (self-reported). Two physicians supervised the process. Vaccinees received vaccination cards and were directed to continue to one of the 6–18 parallel vaccination posts, located behind the check-in station. These posts were equipped with skin disinfectants, Band-Aids, sharps containers, and coolers with prefilled vaccine syringes. Two to four registered nurses staffed each vaccination post. A registered nurse and a physician supervised the vaccinations. Registered nurses prepared single-dose syringes in the cafeteria kitchen, adjacent to the vaccination clinic. Three to 10 persons were involved continuously in preparing the syringes. Syringes were filled from the multidose vial, capped with new needles, and stored in coolers. In a separate room adjacent to the vaccination posts, the local fire department provided staff and equipment for first aid and advanced life support. Two vaccinees needed first aid because of minor injuries, occurring as a result of syncope before or shortly after vaccination. No allergic reactions or injection site infections were reported during the campaign or afterwards. One needlestick injury occurred in a health-care worker.

Public Information

The state health department, county health department, and school administration had difficulty coordinating the release of information to the media and the public. Some elected officials did not accept the targeted approach of the vaccination campaign. Public anxiety resulting in part from inconsistent messages and disagreements among officials hampered efforts to conduct the vaccination campaign as originally planned. The toll-free information phone line registered over 5,000 calls in 7 days. In addition to the affected county health department, health departments from neighboring counties also received hundreds of phone calls each day during the outbreak.

Vaccination Coverage

The target population of 2- to 22-year-old residents of the two neighboring towns was 10,132. A total of 13,535 persons received the vaccine. Of these, 13,148 (97%) were vaccinated during the first 3 days of the campaign; the remaining 387 (3%) were vaccinated by appointment at the health department on January 5–20, 1999. Between 300 and 1,100 vaccinations were given per hour. During maximum workload, 1,100 vaccinations were delivered per hour by approximately 78 workers who staffed the 18 lines. According to the vaccination logs, among the 13,148 persons who received vaccine during the first 3 days, 10,076 (77%) belonged to the target population, 3,065 (23%) lived outside the target area, and 7 (<1%) were older than the target age group (Tables 2 and 3). On the basis of these numbers, vaccination coverage was 99% (10,076/10,132). Vaccination coverage was lower in the age groups 15–20 and 21–22 years than in younger children (Table 3). More than half of all persons in the target group received vaccine on day 1 of the mass campaign; 84% were immunized by the end of day 2 and 96% after day 3 (Table 2).

Among those ages 5–17 with zip codes within the target area (6,699), a sample of 191 (3%) vaccination records were selected, and the names and addresses were compared to the school enrollment database of the county. Among these, 165 (86%) matched in name and birthdate with records in the

Table 2. Vaccinations by day and target area, Putnam County, Florida, 1998–1999

Vaccination dates	Vaccinees within target area	Vaccinees outside target area	Total vaccinees	% of all vaccinees in target area
12/30/98	5,398	229	5,627	96
12/31/98	3,393	656	4,049	84
1/2/99	1,292	2,180	3,472	37
Total first 3 days	10,083	3,065	13,148	77
1/2–20/99	363	24	387	94
Total	10,446	3,089	13,535	77

Table 3. Size of target population and number vaccinated by age group, Putnam County, Florida, 1998–1999

Age group	Estimated target population	No. (%) of target population vaccinated[a]
Approximately 2–4 yrs	1,439	1,435 (100)
5–9 yrs	2,460	2,741 (111)
10–14 yrs	2,513	2,606 (104)
15–20 yrs	2,424	2,288 (94)
21–22 yrs	1,296	1,006 (78)
Total	10,132	10,076 (99)

[a]Figures >100% result from the fact that people outside the target population received vaccination.

school enrollment database. Of these records, 142 (86%) had addresses within the target area in the school enrollment database, while 23 (14%, 95% confidence interval 9% to 19%) had addresses outside the target area. If 14% of the addresses for the 10,076 vaccinees with addresses within the target area were incorrect, approximately 8,664 vaccinees actually were from the target area. The overall estimate for the vaccination coverage for the first 3 days of the campaign would therefore have been 86% (8,664/10,132).

Cost of Intervention

The cost of the public health response to the outbreak amounted to approximately $370,000; 65% of this amount was for the purchase and delivery of vaccine, 20% for personnel costs to administer it, 6% for personnel cost involved in public information, 5% for personnel cost for contact investigation and chemoprophylaxis, and 4% for other expenses (Table 4). The cost for the vaccination campaign alone totaled approximately $329,300 (vaccine, personnel for vaccine administration, and other expenses), resulting in a cost per vaccination of approximately $24.

Discussion

Even accounting for incorrect addresses in the vaccination registers, the vaccination coverage in this campaign was high. Whether the vaccination campaign controlled the outbreak is difficult to prove. The five cases that occurred after the campaign had ended indicate that the population was under continuing exposure to the outbreak strain during the next year. Nevertheless, this low number of subsequent cases suggests that the vaccination campaign probably prevented additional cases that could not have been prevented by chemoprophylaxis alone (19–28). One of these cases was attributed to vaccine failure. In other studies, vaccine efficacy has been estimated to be approximately 85% (4). The size of the unvaccinated population and the number of cases were too small to determine the efficacy of vaccination in this campaign.

The outbreak response was organized within 24 hours after the ACIP criteria were fulfilled and approximately 2 weeks after the first case was reported. This response time compares favorably to that in other vaccination campaigns in response to community-based meningococcal disease outbreaks (3–5,10,14,29). Most mass vaccination campaigns designed in response to community-based outbreaks tend to last more than several weeks or months, which makes a comparison to our results difficult (5,10,29). In England, a mass vaccination campaign conducted in two communities lasted approximately 1 week; 8,320 and 7,660 vaccinations were given in each community, respectively (14). In a meningococcal disease outbreak in Illinois, 125–334 vaccinations were given per hour with jet injectors; however, details were not described (30). Compared with similar reports, providing over 13,000 vaccinations in 3 days and up to 1,100 vaccinations per hour indicates that the intervention was an operational success (5,10,30).

We targeted the vaccination campaign at 2- to 22-year-olds because the vaccine is not effective in children <2 years and because at the time the decision was made none of the patients was >18 years old. We extended the upper age limit to 22 years to include students of the local college since meningococcal disease outbreaks frequently occur in college settings. While the community seemed to accept the upper and lower age limits for the target population, it did not comply well with the geographic restriction. In retrospect, a more targeted approach might be appropriate. However, enforcing a more focused vaccination strategy can be extremely difficult if interventions are needed immediately and under extreme public pressure and fear. The proportion of vaccinees from outside the target area

Table 4. Cost of public health intervention to control meningococcal outbreak, Putnam County, Florida, 1998–1999[a]

Expenditure	Estimated working hrs[b]	Approximate cost (U.S.$)	% of total cost of intervention
Purchase and delivery of the vaccine	N/A	240,500	65
Personnel costs			
Vaccine administration	4,000	74,000	20
Public information	1,200	22,200	6
Contact investigation	1,000	18,500	5
Other	N/A	14,800	4
Total	6,200	370,000	100

[a]NA, not applicable.
[b]Average cost per hour of a public health employee was estimated to be $18.50.

increased each day. Comparison with the school enrollment database showed that 14% of vaccinees had addresses outside the target area. While recent address changes may have been a factor, most of these likely claimed to live in the target area to be eligible for the vaccine.

Public pressure fueled in part by conflicting information released by elected officials became so great that by the afternoon of the 2nd vaccination day restricting vaccination to the target population was impossible. As a result, on the 3rd day most people who were vaccinated lived outside the target area. In an outbreak described by Irwin et al., restricting the campaign to the target population was also difficult (14). Limiting the intervention to only 3 days probably helped to prevent mistargeting of resources, since additional vaccination days would mainly have increased the number of vaccinees from outside the target area.

The effectiveness of chemoprophylaxis in outbreak control has been questioned in previous meningococcal disease outbreaks in Canada and the United Kingdom (9,10). One problem with chemoprophylaxis is that it is effective for a limited period of time. Current recommendations provide guidance for who should receive chemoprophylaxis, but these guidelines can be difficult to implement (7,8,18). Various studies have shown that household contacts are at greater risk of acquiring meningococcal disease and would benefit most from chemoprophylaxis (18,21,22). However, the available evidence does not always indicate who else should receive chemoprophylaxis (6,18,21–28). Twenty-five percent of those who received chemoprophylaxis in this outbreak were not close contacts according to the ACIP definition (7); however, chemoprophylaxis, especially in highly publicized cases, is often given to people who do not fit the definition of a close contact (9,18,19).

The automated toll-free information hot line proved to be an efficient way to respond to the large number of calls from concerned citizens. Confusion might have been avoided if all telephone requests had been directed to only one location sufficiently equipped with phone lines and personnel. We did not brief some of the elected officials early enough to ensure consistent release of information to the public. However, the major problem was the failure of some community leaders to support the targeted approach for vaccination. Similar situations have been described with other vaccination campaigns (12,13,29). The importance of having a close, ongoing relationship between public health and other community leaders cannot be overemphasized. When public health officials have the trust, confidence, and appreciation of other leaders, communicating a unified message is more likely. The circumstances of this outbreak were similar to those expected in the event of a bioterrorist attack, where rapid intervention is required, and the lessons from this outbreak response will be used in Florida to plan for such an event.

The public health response to this outbreak involved considerable expense. In addition to the costs to the health department for personnel and material, the contributions of other state and county agencies, as well as individual volunteers, were substantial. During a school-based vaccination campaign against meningococcal disease in Illinois, approximately $22.50 was spent per vaccination, which is close to our estimate (30). However, during a mass vaccination campaign in the state of Washington, the cost for vaccine administration alone was estimated to be $20 per vaccinee, not including the cost of the vaccine (29). Estimating the cost of future interventions of this type will need to take into account the fact that 50-dose vaccine vials, such as used in this campaign, are no longer available in the United States. The difference in cost is substantial; if we had used 10-dose vials, the cost for the vaccine alone would have increased by approximately 80% to $440,000. This additional cost would have resulted in a cost per vaccinee of $39.

Our decision to implement a vaccination campaign followed ACIP criteria based on epidemiologic and laboratory information. Keeping the public, elected officials, health-care providers, neighboring health departments, and the media informed should be a priority. The vaccination campaign should be confined to the target population to ensure that high vaccination coverage is accomplished early among those most at risk. Based on our experience, providing vaccinations at a single site and in a short time period to maintain control over the operation and conserve resources seems preferable.

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Meteorologic Influences on *Plasmodium falciparum* Malaria in the Highland Tea Estates of Kericho, Western Kenya

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Recent epidemics of *Plasmodium falciparum* malaria have been observed in high-altitude areas of East Africa. Increased malaria incidence in these areas of unstable malaria transmission has been attributed to a variety of changes including global warming. To determine whether the reemergence of malaria in western Kenya could be attributed to changes in meteorologic conditions, we tested for trends in a continuous 30-year monthly malaria incidence dataset (1966–1995) obtained from complete hospital registers at a Kenyan tea plantation. Contemporary monthly meteorologic data (1966–1995) that originated from the tea estate meteorologic station and from global climatology records were also tested for trends. We found that total hospital admissions (malaria and nonmalaria) remained unchanged while malaria admissions increased significantly during the period. We also found that all meteorologic variables showed no trends for significance, even when combined into a monthly suitability index for malaria transmission. We conclude that climate changes have not caused the highland malaria resurgence in western Kenya.

Highland malaria has returned to the tea estates of western Kenya after an absence of nearly 30 years (1–3). Altitude and weather influence malaria epidemiology in highland areas because of the slowing of parasite development within the anopheline vectors at lower temperatures (4). Increased malaria incidence in unstable transmission areas has been variously attributed to changes in land-use patterns (5); population migration (6,7); changes in mosquito vector populations (8); breakdown in provision of health services (9), especially insecticide spraying (10,11); drug resistance (12–16); and meteorologic changes (17,18), particularly global warming (19–25).

We investigated whether climate changes could be implicated in the reemergence of malaria in a unique 30-year malaria and meteorologic time series, collected from the health-care system on a tea plantation in the western highlands of Kenya. Our detailed substudy included site-specific meteorologic and malariometric data from a larger analysis of trends in meteorologic conditions across East Africa from 1911 to 1995 (26–28). Our previous studies have also examined various aspects of the epidemiology of malaria in the Kenyan highlands (29,30).

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Methods

Study Site and Clinical Data

Long-term malaria illness and total hospital admissions data (January 1966–December 1995) exist from a large tea plantation in Kericho, Kenya, which is operated by Brooke Bond Kenya Ltd. (3,31,32). The plantation, located in the western Rift Valley highlands, covers an area of approximately 141 km^2 and ranges from 1,780 to 2,225 m above mean sea level. Epidemic malaria was first recorded on the Kericho tea estates during World War II and was eventually controlled by a combination of mass administration of proguanil and residual insecticide spraying during the late 1940s (2). Currently, the Brooke Bond Kenya Ltd. plantation consists of approximately 20 separate tea estates with a total of 50,000 employees and dependents, who receive their medical care from the company-operated health systems. The company hospital maintains a 24-hour, 7-day clinical admission service for patients who need intensive clinical management. Stained blood smears from patients with suspected malaria are examined; this procedure, in combination with further supportive clinical and laboratory procedures, is used to confirm a primary malaria diagnosis. Case numbers in Kericho can be treated as incidence figures since the population eligible for health care remained at approximately 50,000 during the recording period (32). No centralized preventive chemoprophylaxis, vector control, or bed-net distribution has been implemented since the late 1950s. A substantial minority of the tea estate workers

[1]Dr. Biomndo is deceased.

originate from the holoendemic Lake Victoria area and travel back and forth intermittently to their home areas; however, this travel pattern has been occurring since the road was surfaced in the 1950s and has not changed recently. This study was conducted under a protocol approved by the Kenyan National Ethical Review Committee (SSC 484) and the U.S. Army Office of the Surgeon General (WRAIR 682).

Meteorologic Data

Two meteorologic datasets were compiled. Point locality measurements of mean monthly temperature (°C) and monthly total rainfall (mm) were obtained from the Tea Research Foundation meteorologic station on the Kericho tea estates for the 1966–1995 period. Climate data were also obtained from a global 0.5 x 0.5° (approximately 55 x 55 km [3,025 km^2] at the equator) gridded dataset of monthly terrestrial surface climate for the 1966–1995 period (33,34) (available from: URL: http://www.cru.uea.ac.uk/link). The dataset was used to ensure that results from the single meteorologic station were in agreement with data from a wider geographic area; this procedure also allowed a wider range of climate variables, including temperature extremes, to be tested. Primary variables of precipitation (mm), mean temperature (°C), and diurnal temperature range (°C) were available and interpolated from extensive meteorologic station data by using angular distance-weighted averaging of anomaly fields. The secondary variable of vapor pressure was also provided, interpolated where available, and calculated from primary variables, when the coverage of meteorologic stations was insufficient. Minimum and maximum monthly temperature estimates were created by subtracting or adding, respectively, half the diurnal temperature range from mean monthly temperature. Time series were derived by using an extraction routine developed in ENVI (Research Systems Inc., Boulder, CO) with georeferencing information for Kericho (0.33°S, 35.37°E), obtained from Encarta (Microsoft, Seattle, WA).

To investigate whether a combination of meteorologic conditions was changing and thus facilitating the resurgence of malaria, we also categorized months as suitable for *Plasmodium falciparum* transmission if they had a mean monthly temperature exceeding 15°C (since temperatures experienced by the indoor resting *Anopheles gambiae* vectors are likely to be 3°C–5°C higher) and monthly rainfall totals exceeding 152 mm (1,4) by using the gridded climatology data. The numbers of suitable months for transmission were summed, totaled for each year, and tested for the 1966–1995 period.

Statistical Analyses

To test for trends in the climate and malaria suitability time series, we estimated the following regression equation:

$$\Delta y_t = \alpha + \beta t + \gamma y_{t-1} + \sum_{i=1}^{p} \delta_i \Delta y_{t-1} + \sum_{j=1}^{12} \mu_j d_j + \varepsilon_t \quad (1)$$

where y is the variable of interest; α, β, γ, and μ_j's are regression parameters; ε_t is a normally distributed error term with mean zero; and t is a deterministic time trend. The centered dummy variables d_j model the monthly seasonal variations in climate. The coefficients μ_j sum to zero. Δ is the first difference operator. The lagged values of the dependent variable model the serial correlation in the dependent variable. We chose the number of lags, p, using the adjusted R-square statistic. The maximal number of lags p considered was 24.

If the time series y can be characterized as the sum of a stationary stochastic process and a linear time trend, then the appropriate test for the trend is a t test on β in (1). If the series is a random walk, however, or a more complex stochastically trending process, the critical levels for the distribution of the t score in this regression are much greater than usual (35), and alternative tests should be employed. Since many climate time series contain a stochastically trending component (36), the nature of the series must be explored before testing for climate change. This methodology issue complicates the evaluation of the significance of trends established with standard regression procedures often used in such studies.

If $\gamma = 0$ (a unit root in the autoregressive process) and $\beta = 0$, then y is a random walk. The random walk may also have a deterministic drift term ($\alpha \neq 0$). In either case, however, the series is nonstationary, and classical regression inference does not apply. The nonstandard distributions of α, β, and γ have been tabulated by Dickey and Fuller (37,38). We first tested for the presence of a unit root by evaluating the t statistic for γ against its nonstandard distribution. The critical value for this so-called Augmented Dickey-Fuller at the 5% level is -3.45. Values of the t statistic for γ more negative than this critical value indicate that the series is not a random walk and vice versa. If the null hypothesis is rejected, then the t statistics associated with α and β are normally distributed. If the unit root hypothesis is accepted, then these statistics also have nonstandard distributions. The correct test for a trend is then the t test on α in (1) with the omission of the linear trend. The test's critical value at the 5% significance level is 2.54. The results of these tests are presented in the Table.

We also regressed temperature and rainfall data from the meteorologic station at Kericho on the same variables from the interpolated climatology (33,34) by using a variety of formulations including levels, logarithms, and a regression adjusted for heteroscedasticity. We then tested whether the slope coefficients were significantly different from unity, which should not be the case if the gridded dataset is a good proxy for the climate at Kericho.

Results

During the period 1966–1995, malaria incidence increased significantly ($p=0.0133$) while total (i.e., malarial and other) admissions to the tea estate hospital showed no significant change (Table and Figure 1a,b). Measurements of mean monthly temperature and total monthly rainfall also showed no significant changes (Table and Figure 1c,d). Similar results were shown by the climatology data interpolated from a wider area. Mean, maximum, and minimum monthly temperatures;

Table. Trend of malaria, climate, and malaria suitability variables, Kericho tea estates, 1966–1995[a,b]

Variable	p	ADF[c]	b	t	p value[c]	ta	Q	Sig. Q
Malaria incidence	5	-4.00	0.0238	2.49	0.0133	0.1801	58.7394	0.0097
Total admissions	6	-2.76	-0.0069	-0.28	0.7820	-0.4151	30.9302	0.7083
Tmean met. stat. (°C)	8	-3.41	0.0004	1.76	0.0799	-0.0211	40.8630	0.2653
Rain met. stat. (mm)	1	-11.91	-0.0202	-0.52	0.6066	-0.0074	43.3753	0.1858
Tmean clim. (°C)	1	-7.51	0.0035	1.60	0.1103	-0.0980	46.6888	0.1094
Tmax clim. (°C)	24	-4.66	0.0070	1.68	0.0935	0.0592	22.6634	0.9592
Tmin clim. (°C)	1	-8.36	0.0038	1.55	0.1233	-0.1944	45.1424	0.1412
Precipitation clim. (mm)	1	-11.70	-0.0098	-0.36	0.7205	-0.0745	34.2984	0.5497
Vapor pressure clim. (hPa)	1	-8.37	0.0038	1.66	0.0974	-0.1829	45.5674	0.1318
Garnham suitability (mo)[d]	4	-4.21	-0.0380	**-0.89**	0.3850	-0.4488	5.6658	0.7729

[a]Tmean, the mean monthly temperature; Tmax, the mean of maximum monthly temperatures; Tmin, the mean of minimum monthly temperatures; met. stat., meteorologic station data from the Kericho tea estate; clim., data derived from the global gridded climatology dataset (33,34).
[b]Figures in bold denote significance at the 5% level. p is the number of lagged differenced dependent variables selected.
[c]ADF, the Augmented Dickey-Fuller t-test for γ=0. The 5% critical value is -3.45. Exact p values are not available for ADF and $\tau\alpha$ statistics. The distribution of the t statistic for the slope parameter β has the standard t distribution under the assumption that γ<0. $\tau\alpha$ is the t statistic for the intercept term in the autoregression without a linear time trend. This test is the appropriate one for a trend if γ=0. Its 5% critical value is 2.54. The Q statistic is a portmanteau test for general serial correlation and is distributed as chi square (39).
[d]Garnham suitability (1,4) refers to the number of months with a mean monthly temperature exceeding 15°C and monthly rainfall totals exceeding 152 mm (when the gridded climatology data are used). These data are therefore annual data, whereas all other time-series are monthly observations.

precipitation; and vapor pressure all demonstrated no significant trends (Table; Figure 2a,b,c). Moreover, the interpolated climatology data, when transformed into month of malaria transmission suitability (1,4), again showed no significant changes (Table; and Figure 2d).

Results were very similar, though significance levels varied, between the three formulations of the regression model that compared the local meteorologic station data and those from the interpolated climatology data (33,34). The coefficient for the regression of the meteorologic station rainfall data on the interpolated climatology precipitation data is in every case not significantly different from unity. Significance levels are 10% for the model in levels, 18% for the heteroscedasticity-adjusted model, and 96% for the logarithmic model. In the regression of the two temperature series, however, the coefficient is significantly different from unity in every case, as is a joint test statistic for the two slope coefficients.

Discussion

The resurgence of *P. falciparum* malaria in the East African highlands (3,8,18,26, 40–44) has led several researchers to speculate that climate change is a predominant cause (23,45–50). On the basis of these studies, which have been disputed by experts in vector-borne disease biology (10,27–29, 51,52), and some biological modeling, which has been robustly criticized (53), the International Panel on Climate Change has recently concluded with "medium-to-high confidence" that there will be a net increase in the range and incidence of malaria (49); the results of our work do not support these conclusions.

Malaria incidence increased significantly (p=0.0133) during the 1966–1995 period, while total admissions remained unchanged. Besides an increase in local malaria transmission, two other factors may have influenced the increase in malaria hospitalizations. An increase in malaria severity indicated by an increased case-fatality rate (from 1.3% in the 1960s to 6% in the 1990s) is most likely linked to chloroquine resistance, which we believe to be the probable cause of much of the overall increase in malaria transmission (32). Travel to and from the Lake Victoria region by a minority of the tea estate workers also exerts an upward influence on malaria transmission in Kericho since such travel increases the numbers of workers asymptomatically carrying gametocytes, which infect

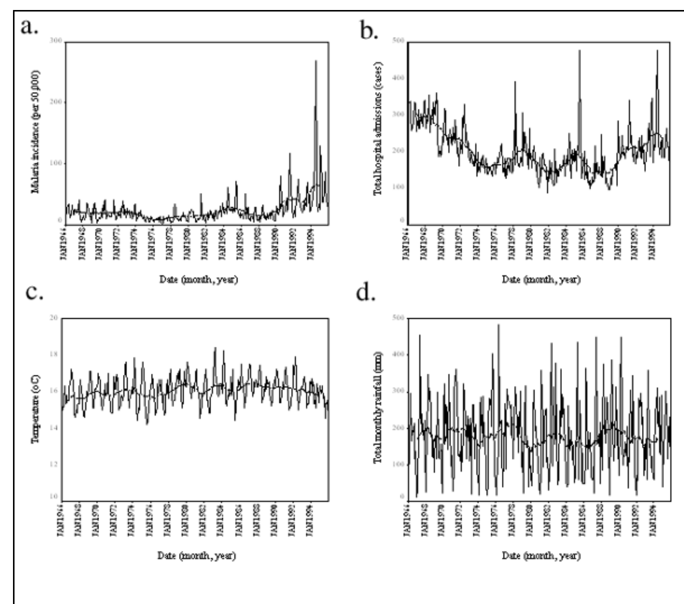

Figure 1. Malaria, hospital admissions, and meteorologic station data, Kericho tea estate, 1966–1995. Malaria incidence (a) total hospital admissions (b) mean monthly temperature (c) and total monthly rainfall (d) are all plotted with a 25-point (month) moving average (bold) to show the overall movement in the data. The significance of these movements is presented in Table.

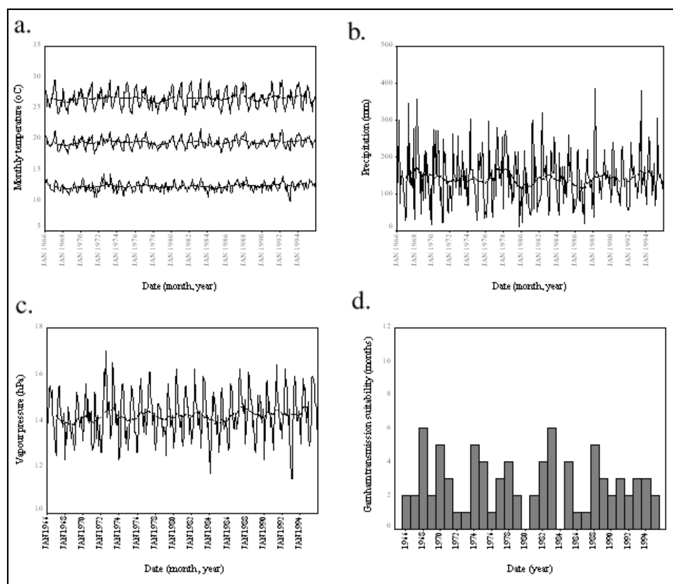

Figure 2. Climate and malaria suitability data for the Kericho area from the global gridded climatology data, including meteorologic and malaria suitability time series. Minimum (bottom), mean (middle) and maximum (top) monthly temperature (a) total monthly precipitation (b) and mean vapor pressure (c) are all plotted with a 25-point (month) moving average (bold) to show the overall movement in the data. The number of months per year suitable for malaria transmission (d) are also plotted. Suitability was determined if rainfall exceeded 152 mm and temperature exceeded 15°C in any month (1,4). The significance of these movements is presented in Table.

mosquitoes for further human infection. This complex topic is the subject of a future publication.

All climate variables, whether from the Kericho tea estate meteorologic station or the pixel covering Kericho in the global climatology dataset showed no significant trends, despite the fact that equivalence tests showed some significant differences between the temperature time series—findings that are in agreement with a broader geographic analysis of East African data from 1911 to 1995 (26) and lend support to the appropriateness of interpolated climate data for use in these investigations. We also think that, when examining trends in meteorologic phenomena, epidemiologists should use more robust statistical techniques for the reasons outlined in the methods. The results of this detailed examination of coincident empirical data do not support the widespread, recent speculation regarding malaria resurgences in response to climate change. No aspect of climate has changed significantly—neither the temperature extremes (maximum and minimum) nor the periods when meteorologic data were transformed into months when malaria transmission is possible. Further study has also shown that variability in these meteorologic variables, independent of any longer term trends, has decreased (54). We must therefore look elsewhere for the causes of these resurgences (27,28,32). These factors are likely to vary. In Kericho, however, increased chloroquine resistance has been strongly argued to be the cause, since all other relevant environmental and sociologic factors are unchanged (32).

The attraction of the global warming hypothesis as an explanation of highland malaria is the existence of a continental trend toward global warming coincident with a trend toward increasing malaria incidence in several parts of Africa, ranging from Senegal (13,14) to Madagascar (10). Where such malaria increases have been examined in detail, however, alternative explanations such as discontinuation of anti-vector measures in Madagascar (10) or chloroquine resistance in Senegal appear to be more likely causes (13,14). Malaria epidemiology is greatly influenced by a range of local factors, making a consistent continent-wide explanation seem unlikely (28,52).

We do not argue that meteorologic conditions have no immediate impact on the seasonal dynamics and incidence of malaria or that climate change is probably not an important future concern in public health. Rather we urge some caution in the interpretation of synonymous changes in climate over wider areas and local changes in malaria incidence.

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Antimicrobial Resistance of *Escherichia coli* O26, O103, O111, O128, and O145 from Animals and Humans

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Susceptibilities to fourteen antimicrobial agents important in clinical medicine and agriculture were determined for 752 *Escherichia coli* isolates of serotypes O26, O103, O111, O128, and O145. Strains of these serotypes may cause urinary tract and enteric infections in humans and have been implicated in infections with Shiga toxin–producing *E. coli* (STEC). Approximately 50% of the 137 isolates from humans were resistant to ampicillin, sulfamethoxazole, cephalothin, tetracycline, or streptomycin, and approximately 25% were resistant to chloramphenicol, trimethoprim-sulfamethoxazole, or amoxicillin-clavulanic acid. Approximately 50% of the 534 isolates from food animals were resistant to sulfamethoxazole, tetracycline, or streptomycin. Of 195 isolates with STEC-related virulence genes, approximately 40% were resistant to sulfamethoxazole, tetracycline, or streptomycin. Findings from this study suggest antimicrobial resistance is widespread among *E. coli* O26, O103, O111, O128, and O145 inhabiting humans and food animals.

The emergence and dissemination of antimicrobial resistance in bacteria has been well documented as a serious problem worldwide (1). Selective pressure favoring antimicrobial-resistant phenotypes is applied whenever antimicrobials are used, including treating disease in clinical medicine and preventing disease and promoting growth in animal husbandry. As a consequence, antimicrobial-resistant bacteria are selected for, thereby posing a critical public health threat in that antimicrobial treatment efficacy may be reduced.

Escherichia coli are facultative anaerobes in the normal intestinal flora of humans and animals (2,3); however, pathogenic strains of these bacteria are an important cause of bacterial infections. In humans, these strains are the foremost cause of urinary tract infections (4), as well as a major cause of neonatal meningitis (5), nosocomial septicemia, and surgical site infections (6). Infection with Shiga toxin–producing *E. coli* (STEC) may also result in complications including thrombocytopenic purpura, severe hemorrhagic colitis, and hemolytic uremic syndrome (7). While therapeutic options vary depending on the type of infection, antimicrobials including trimethoprim-sulfamethoxazole, fluoroquinolones, and third-generation cephalosporins are generally recommended for treating infections caused by *E. coli* other than STEC (6). In contrast, because these antimicrobials may increase levels of free Shiga toxin in vivo, thus facilitating disease progression, the usefulness of antimicrobials in treating STEC infection remains less clear (6,8).

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Recent reports have suggested the use of tetracyclines, sulfa drugs, cephalosporins, and penicillins to be a major factor in the emergence and dissemination of antimicrobial-resistant *E. coli* (9–14). However, a relative paucity of information exists regarding antimicrobial resistance in *E. coli* from nonhospital sources, especially those from animal sources. In this study, antimicrobial susceptibility profiles were determined for *E. coli* isolates of serotypes O26, O103, O111, O128, and O145. Strains of these serotypes may cause urinary tract and enteric infections in humans and have been implicated in infections with STEC (15–19). The isolates were originally gathered from diverse sources, including food animals, companion animals (i.e. dogs, cats, and rabbits), and humans. Our primary objective was to characterize the extent of antimicrobial resistance in these *E. coli* serotypes from agricultural and clinical settings.

Methods

Bacterial Strains

We included 752 *E. coli* isolates from the collection of The Pennsylvania State University's *E. coli* Reference Center in the study (Table 1); this center provides characterization of *E. coli* isolates submitted from outside sources. Sixty-eight isolates from humans were submitted to the *E. coli* Reference Center from 9 U.S. states, 45 from Saudi Arabia, 13 from Argentina, 4 from Canada, 3 from Mexico, 3 from Zambia, and 1 from Singapore. Two hundred forty-eight isolates from cattle were submitted from Michigan, 56 from Iowa, 33 from Pennsylvania, 65 from 13 other U.S. states, and 2 from Canada. Fifty-one isolates from turkeys were submitted from 13

Table 1. Source of isolation, genotype, serotype, and year of isolation of *Escherichia coli* isolates

		Genotype		Serotype					Year				
Source	No. isolates	STEC[a]	Other *E. coli*	O26	O103	O111	O128	O145	1976–1980	1981–1985	1986–1990	1991–1995	1996–2000
Human	137	37	100	19	23	37	53	5	0	19	4	87	27
Cow	408	140	268	230	65	60	18	35	15	16	37	60	280
Turkey	51	3	48	3	9	38	0	1	0	3	28	2	18
Chicken	49	0	49	14	21	10	3	1	5	5	21	5	13
Pig	26	3	23	9	7	2	7	1	10	6	6	1	3
Nonfood animals	81	12	69	11	43	0	13	14	0	19	30	5	27
Totals	752	195	557	286	168	147	94	57	30	68	126	160	368

[a]STEC, Shiga toxin–producing *E. coli*, determined by the presence of *stx1* and/or *stx2*.

U.S. states. Forty-five isolates from chickens were submitted from 10 U.S. states, 2 from Canada, and 2 from India. Twenty-two isolates from swine were submitted from 7 U.S. states, 3 from South Korea, and 1 from India. Seventy-four isolates from nonfood animals were submitted from 20 U.S. states, 5 from Paraguay, and 2 from Hungary. We classified nonfood animals as those not commonly used in food production, including rabbits (19 *E. coli* isolates), hamsters (8 isolates), deer (7 isolates), horses (7 isolates), dogs (7 isolates), alpacas (5 isolates), okapi (4 isolates), parrots (4 isolates), sheep (4 isolates), antelope (4 isolates), mice (3 isolates), seagulls (2 isolates), a cat (1 isolate), a goat (1 isolate), a llama (1 isolate), a marmoset (1 isolate), a mink (1 isolate), a rat (1 isolate), and a turtle (1 isolate).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of all isolates was done with broth microdilution using the PASCO MIC/ID system (Becton, Dickinson and Company, Sparks, MD). Testing was done according to manufacturer's instructions and according to guidelines developed by the National Committee for Clinical Laboratory Standards (NCCLS) (20). Tested antimicrobials, dilution ranges, and resistance breakpoints are listed in Table 2. Ceftiofur- and cefoxitin-resistant isolates were further examined for production of extended-spectrum-β-lactamases (ESBLs) with disk diffusion according to NCCLS standards (21).

Detection of Virulence Genes

Isolates were grown at 37°C overnight on veal infusion agar (Becton, Dickinson and Company). A loopful of culture was resuspended in 200 µL of distilled water, incubated at 99°C for 15 min, and centrifuged at 12,000 x *g* for 2 min. The supernatant was used as a template for amplification of Shiga toxin genes (*stx1* and *stx2*), the intimin gene (*eae*), and the enterohemolysin A gene (*hlyA*) through multiplex polymerase chain reaction (PCR) (22). Primers described by Witham et al. (23) and Paton (24) were used for amplification of *stx1* and *stx2*, respectively; those described by Gannon et al. (25) were used for amplification of *eae*; and those described by Fagan et al. (26) were used for amplification of *hlyA*. Each 11-µL PCR contained 37.5 ng *stx1* primers, 15 ng *stx2* primers, 15 ng *eae* primers, 75 ng *hlyA* primers, 0.18mM each deoxyribonucleotide, 4.0mM $MgCl_2$, 50mM Tris-HCl (pH 8.3), 275 ng bovine serum albumin, 2% sucrose, 0.1mM Cresol Red (Idaho Tech-

Table 2. Class, dilution range, and resistant breakpoints of tested antimicrobials[a]

Class or antimicrobial	Dilution range tested (µg/mL)	NCCLS resistance breakpoint (µg/mL)
Cephalosporins		
Cefoxitin	1–32	32
Ceftiofur	1–16	8[b]
Ceftriaxone	0.06–64	64
Cephalothin	1–32	32
Penicillins		
Amoxicillin-clavulanic acid	0.25/0.12–32/16	32/16
Ampicillin	0.25–32	32
Sulfonamides and potentiated sulfonamides		
Sulfamethoxazole	32–512	512
Trimethoprim-sulfamethoxazole	0.06/1.19–4/76	4/76
Phenicols		
Chloramphenicol	1–32	32
Quinolones and fluoroquinolones		
Ciprofloxacin	0.004–8	4
Nalidixic acid	2–256	32
Aminoglycosides		
Gentamicin	0.25–16	16
Streptomycin	1–256	64
Tetracycline	1–16	16

[a]NCCLS, National Committee for Clinical Laboratory Standards. Antimicrobial susceptibility testing was performed according to NCCLS standards (20). *Escherichia coli* (ATCC 25922 and ATCC 35218), *Enterococcus faecalis* (ATCC 51299), and *Pseudomonas aeurigonosa* (ATCC 27853) were used as quality controls.
[b]NCCLS breakpoint not established for *E. coli*.

nology, Inc., Salt Lake City, UT), and 0.4 U *Taq* DNA polymerase (PGC Scientifics Corp., Gaithersburg, MD). Reaction contents were cycled as described (11) after which products were electrophoresed in 1% agarose gels at 200 V for 30 min and visualized under ultraviolet light. *E. coli* O157:H7 (ATCC 43895) was the positive control for all reactions.

Results

Antimicrobial Resistance Compared to Isolation Source

Of the isolates in this study, the highest frequencies of antimicrobial-resistant phenotypes were observed for *E. coli* isolates from humans and turkeys (Figure 1). Fifty-nine percent of isolates from humans were resistant to sulfamethoxazole, 59% to streptomycin, 56% to ampicillin, 56% to tetracycline, 50% to cephalothin, 38% to trimethoprim-sulfamethoxazole, 34% to chloramphenicol, and 18% to amoxicillin-clavulanic acid (Figure 1A). Eighty-four percent of isolates from turkeys were resistant to sulfamethoxazole, followed by 82% to streptomycin, 71% to tetracycline, 49% to ampicillin, 39% to cephalothin, 28% to amoxicillin-clavulanic acid, 24% to gentamicin, and 20% to nalidixic acid (Figure 1B). Nalidixic acid-resistant isolates from turkeys were found to have ciprofloxacin MICs ranging from 0.12 to >8 µg/mL, whereas each of the nalidixic acid-susceptible isolates from these animals were found to have ciprofloxacin MICs of 0.03 µg/mL or less (data not shown).

Resistance profiles among isolates from cattle, chicken, and swine were largely similar to each other (Figure 1). Fifty percent of isolates from cattle were resistant to streptomycin, followed by 47% to tetracycline, 46% to sulfamethoxazole, and 15% to ampicillin (Figure 1C). Seventy-one percent of isolates from chickens were resistant to streptomycin, followed by 63% to tetracycline, 53% to sulfamethoxazole, 20% to gentamicin, 16% to trimethoprim-sulfamethoxazole, and 12% to ampicillin (Figure 1D). Eighty-one percent of isolates from swine were resistant to tetracycline, followed by 62% to streptomycin, 31% to sulfamethoxazole, and 27% to ampicillin (Figure 1E).

Resistance frequencies were lowest for isolates from non-food animals (Figure 1F); however, 25% were resistant to streptomycin, 20% to sulfamethoxazole, and 18% to tetracycline. Of these streptomycin-, sulfamethoxazole-, and tetracycline-resistant isolates, 76%, 82%, and 67%, respectively, were from companion animals.

Of 174 isolates resistant to ampicillin, 73% were resistant to streptomycin and tetracycline. Of 23 isolates resistant to cefoxitin, 91% were resistant to amoxicillin-clavulanic acid. Each of the five ceftiofur-resistant isolates was resistant to cefoxitin and amoxicillin-clavulanic acid. Based on NCCLS interpretive criteria for confirmatory ESBL testing (21), none of the ceftiofur- or cefoxitin-resistant isolates exhibited phenotypes consistent with ESBL production.

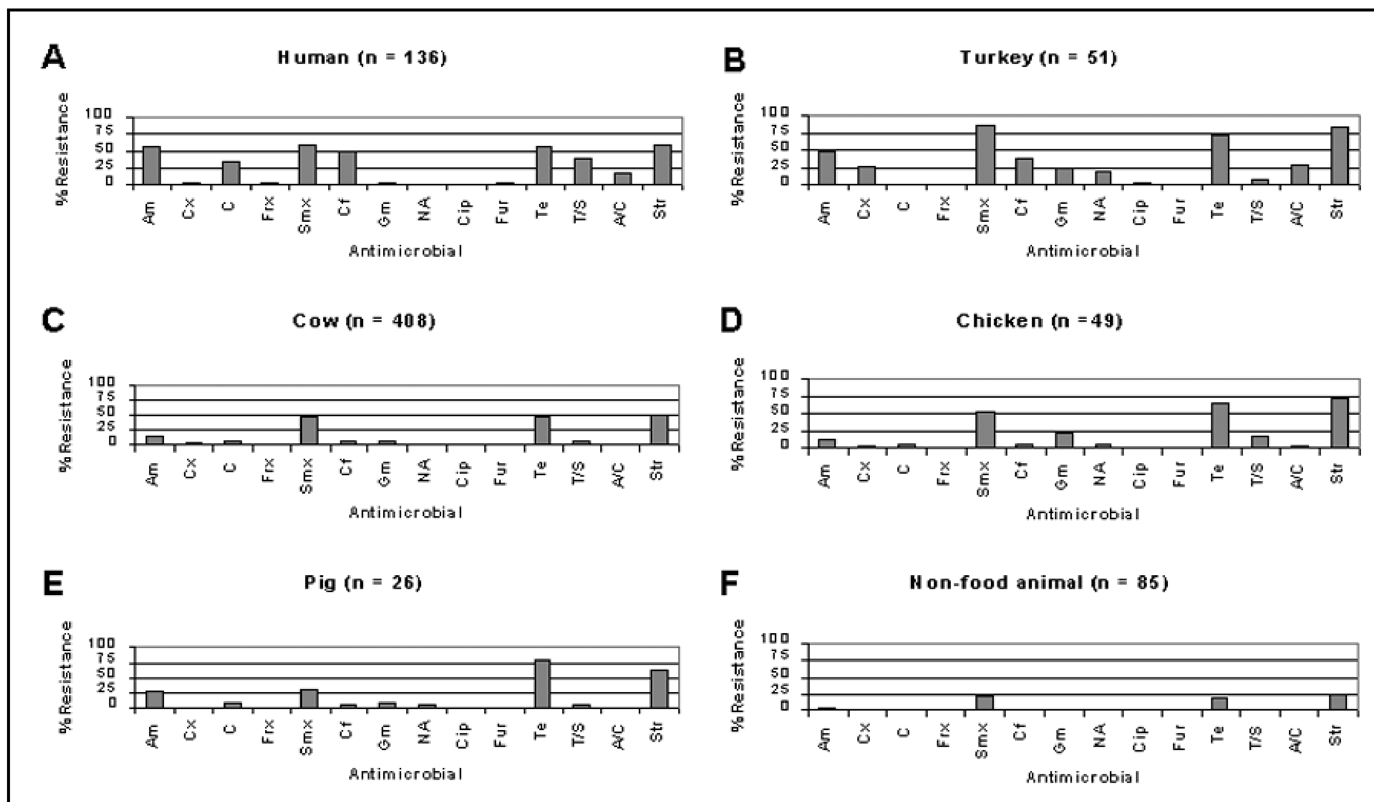

Figure 1. Comparison of antimicrobial resistance frequencies for *Escherichia coli* isolates from different sources. Am, ampicillin; Cx, cefoxitin; C, chloramphenicol; Frx, ceftriaxone; Smx, sulfamethoxazole; Cf, cephalothin; Gm, gentamicin; NA, nalidixic acid; Cip, ciprofloxacin; Fur, ceftiofur; Te, tetracycline; T/S, trimethoprim-sulfamethoxazole; A/C, amoxicillin-clavulanic acid; Str, streptomycin.

Presence of Virulence Genes and Antimicrobial Resistance in STEC

Based on the presence of *stx1* and *stx2*, 26% of the isolates were characterized as STEC. Of these, 89% contained *stx1* only, 2% contained *stx2* only, and 9% contained both. Eighty-one percent of STEC possessed *eae* and *hlyA*, 7% *eae* only, and 7% *hlyA* only. Of isolates that were not characterized as STEC, 34% possessed *eae* and *hlyA*, 2% *eae* only, and 24% *hlyA* only (data not shown).

The highest frequency of STEC was among isolates from cattle, in which 34% were characterized as STEC, followed by 27% of isolates from humans, 14% of isolates from nonfood animals, 12% of isolates from swine, and 6% of isolates from turkeys. None of the isolates from chickens were characterized as STEC.

Of *E. coli* isolates from cattle, resistance frequencies were generally similar between STEC and other *E. coli*, respectively, with the exception of ampicillin (26% vs. 8%), chloramphenicol (14% vs. 4%), cephalothin (14% vs. 3%), and trimethoprim-sulfamethoxazole (11% vs. 2%), in which resistance frequencies were noticeably higher (Figure 2A). In contrast, of isolates from humans, resistance frequencies were generally lower among STEC isolates compared with other *E. coli* (Figure 2B). Specifically, resistance frequencies were lower in STEC compared with other *E. coli*, respectively, for ampicillin (14% vs. 71%), chloramphenicol (5% vs. 44%), sulfamethoxazole (30% vs. 68%), cephalothin (11% vs. 64%), tetracycline (32% vs. 63%), trimethoprim-sulfamethoxazole (8% vs. 48%), amoxicillin-clavulanic acid (5% vs. 22%), and streptomycin (32% vs. 67%).

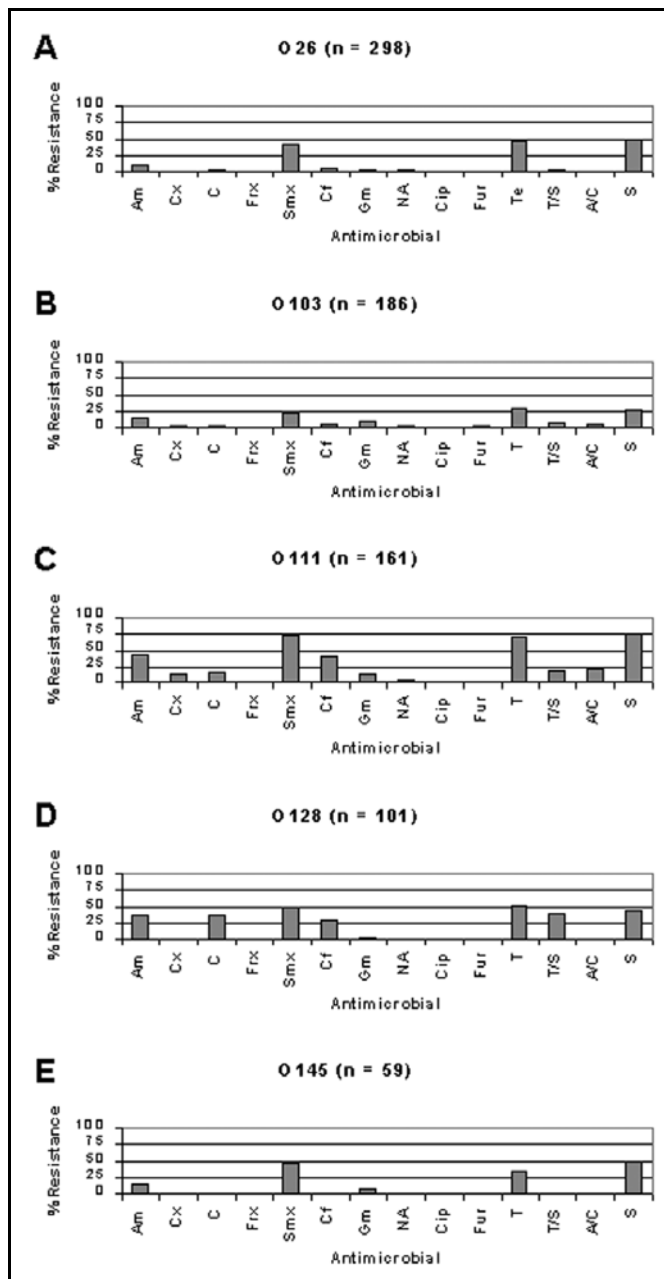

Figure 2. Comparison of antimicrobial resistance frequencies between Shiga toxin-producing *Escherichia coli* (STEC) and other *E. coli*. Of isolates from humans, resistance frequencies were generally lower for STEC compared with other *E. coli* (A). In contrast, of isolates from cattle, resistance frequencies were similar between STEC and other *E. coli* (B). Am, ampicillin; Cx, cefoxitin; C, chloramphenicol; Frx, ceftriaxone; Smx, sulfamethoxazole; Cf, cephalothin; Gm, gentamicin; NA, nalidixic acid; Cip, ciprofloxacin; Fur, ceftiofur; Te, tetracycline; T/S, trimethoprim-sulfamethoxazole; A/C, amoxicillin-clavulanic acid; Str, streptomycin.

Discussion

Of the 752 *E. coli* isolates characterized in this study, approximately half displayed resistance to one or more antimicrobials, including penicillins, sulfonamides, cephalosporins, tetracyclines, and aminoglycosides. These data are in accord with multiple previous studies suggesting use of these drugs has been a key factor in the emergence of antimicrobial-resistant *E. coli* (10–13,27,28). In addition, several other findings from this study are noteworthy in terms of their public health importance.

Approximately 40% of *E. coli* from humans was resistant to trimethoprim-sulfamethoxazole. Because this drug combination is recommended for treating a range of human infections, including complicated urinary tract infections, acute uncomplicated cystitis, and pyelonephritis (6), *E. coli* isolates should be monitored for further dissemination of trimethoprim-sulfamethoxazole resistance. Virtually all trimethoprim-sulfamethoxazole-resistant isolates from this study, however, were susceptible to ciprofloxacin and ceftriaxone, both of which are important antimicrobials for treating infections caused by trimethoprim-sulfamethoxazole-resistant *E. coli*.

Ceftiofur is the sole extended-spectrum cephalosporin approved for use in food animals in the United States, and it is not approved for use in human clinical medicine (29). The observation, therefore, that two isolates from humans displayed resistance to ceftiofur suggests the transfer of resistant *E. coli* from food animals to humans (28,30,31). However, because these two isolates also displayed resistance or decreased susceptibility to other β-lactam antimicrobials, including ampicillin, amoxicillin-clavulanic acid, cephalothin, cefoxitin, and ceftriaxone, ceftiofur-resistance in these isolates might have resulted from of β-lactam use in clinical medicine.

Similarly, the relatively high number of cefoxitin-resistant isolates from turkeys compared to those from other sources may be attributable to β-lactam antimicrobial use in turkey production. While, based on confirmatory tests, none of the ceftiofur- or cefoxitin-resistant isolates identified in this study yielded phenotypes consistent with ESBL production, these isolates may have produced plasmid-mediated AmpC-like β-lactamases, similar to those described for other *E. coli* and *Salmonella* isolated from food animals (28–30). Consequently, work is ongoing to further characterize the genetic basis of β-lactam resistance in these isolates.

The observation that 20% of *E. coli* isolates from turkeys were resistant to nalidixic acid (concomitant with increased MICs for ciprofloxacin) is important considering fluoroquinolones are used to treat a range of *E. coli* infections in humans (6). This finding, similar to those of previous reports (14,32,33), may be largely attributable to fluoroquinolone use in turkeys. The impact of fluoroquinolones such as enrofloxacin in turkey production on the emergence of quinolone- and fluoroquinolone-resistant bacteria should continue to be monitored.

Virtually all *E. coli* isolates from nonfood animals were susceptible to each of the antimicrobials tested. Notable exceptions, however, were isolates from dogs, cats, and rabbits. While these data yield preliminary evidence suggesting companion animals may be an important reservoir of antimicrobial-resistant *E. coli* of these serotypes, additional studies are required to more clearly define the impact of antimicrobial use in companion animal medicine on the emergence of antimicrobial-resistant *E. coli.*

STEC-associated virulence genes, including *stx1*, *stx2*, *eae*, and *hlyA*, were detected primarily in isolates from humans and cattle. Differences in pathogenicity of STEC for these two hosts may explain why STEC from humans had a higher frequency of antimicrobial resistance compared to STEC from cattle. Specifically, because in human clinical medicine antimicrobials are likely used less often to treat STEC infections compared with other *E. coli* infections (6,8), frequencies of antimicrobial resistance for STEC were generally lower than those for other *E. coli* from humans. In contrast to humans, cattle are asymptomatic carriers of STEC (34); thus the decision to use antimicrobials in cattle production does not depend upon whether or not these bacteria are present. Accordingly, antimicrobial resistance frequencies of STEC and other *E. coli* from cattle were largely similar to each other.

The multiple antimicrobial-resistant phenotypes observed in this study may have resulted from the spread of mobile genetic elements. For example, the observation that nearly 75% of ampicillin-resistant *E. coli* isolates were also resistant to streptomycin and tetracycline suggests resistance genes for these drugs are linked on plasmids. Moreover, the widespread resistance to sulfamethoxazole implies the presence of class I integrons, which are also important in conferring resistance to multiple antimicrobials (35). Research is continuing to further characterize sulfamethoxazole-resistant *E. coli* for the presence of these mobile genetic elements.

Because the isolates from this study were to a large extent unevenly distributed as to source of isolation versus year of isolation, analyzing resistance trends over time was not possible. Likewise, meaningful analysis of antimicrobial resistance in relation to geographic origin or to serotype was not possible. Long-term prospective studies examining isolates from defined geographic locales are required to more precisely detect temporal and spatial differences in antimicrobial resistance in strains of *E. coli*.

Emergence and dissemination of antimicrobial resistance in *E. coli* strains of serotypes O26, O103, O111, O128, and O145 may complicate treatment of certain urinary tract and enteric infections in humans and animals. Data from this study did not demonstrate a steadfast link between antimicrobial use in any particular venue and development of antimicrobial resistance among these *E. coli* isolates. The data did, however, suggest that antimicrobial use in clinical medicine and in agriculture was important in the selection of antimicrobial-resistant phenotypes. Continued surveillance of *E. coli* collected from agricultural and clinical settings, including the food production continuum, is merited to identify emerging antimicrobial-resistant phenotypes.

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At the time this article was written, Dr. Schroeder was a faculty research associate at the University of Maryland, under the direction of Dr. Meng. He is now a risk analyst with the United States Department of Agriculture Food Safety and Inspection Service in Washington, D.C. Together with the coauthors, his research interests include factors affecting antimicrobial resistance of food-borne bacterial pathogens.

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Genetic Analysis of Viruses Associated with Emergence of Rift Valley Fever in Saudi Arabia and Yemen, 2000-01

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The first confirmed Rift Valley fever outbreak outside Africa was reported in September 2000, in the Arabian Peninsula. As of February 2001, a total of 884 hospitalized patients were identified in Saudi Arabia, with 124 deaths. In Yemen, 1,087 cases were estimated to have occurred, with 121 deaths. Laboratory diagnosis of Rift Valley fever virus (RVFV) infections included virus genetic detection and characterization of clinical specimens by reverse transcription-polymerase chain reaction, in addition to serologic tests and virus isolation. Genetic analysis of selected regions of virus S, M, and L RNA genome segments indicated little genetic variation among the viruses associated with disease. The Saudi Arabia and Yemen viruses were almost identical to those associated with earlier RVF epidemics in East Africa. Analysis of S, M, and L RNA genome segment sequence differences showed similar phylogenetic relationships among these viruses, indicating that genetic reassortment did not play an important role in the emergence of this virus in the Arabian Peninsula. These results are consistent with the recent introduction of RVFV into the Arabian Peninsula from East Africa.

Rift Valley fever (RVF) (caused by Rift Valley fever virus [RVFV], family *Bunyaviridae*) is an emerging epidemic disease of humans and livestock, as well as an important endemic problem in sub-Saharan Africa. The virus is transmitted to livestock and humans by the bite of infected mosquitoes or exposure to tissues or blood of infected animals. Massive epizootics are typically observed in livestock during times of unusually high and sustained rainfall because of the presence of breeding sites and overabundance of adult competent mosquito vectors (1). Infections caused by RVFV are characterized by severe disease and abortion in livestock, particularly sheep and cattle. Persons in the epidemic region are at high risk for RVFV infection, potentially leading to thousands of human cases. Humans infected with RVFV typically have self-limited febrile illness, but retinal degeneration (5–10%), hemorrhagic fever (<1%), or encephalitis (<1%) may also develop (2).

We report the first confirmed outbreak of RVF outside Africa, in the Kingdoms of Saudi Arabia and Yemen. On September 10, 2000, the Ministry of Health in Saudi Arabia began to receive reports of unexplained hemorrhagic fever in humans near the Saudi-Yemeni border, with associated animal deaths and abortions. Patient samples from the outbreak were sent to the Centers for Disease Control and Prevention (CDC), where laboratory analysis confirmed the cases as being caused by RVFV. Genetic analysis was performed on all three viral RNA segments from human clinical samples, and the sequences were compared with previously characterized RVFV isolates to determine their genetic relatedness and geographic distribution.

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Materials and Methods

Clinical Specimens

On September 15, 2000, acute-phase sera from four seriously ill, hospitalized patients with unexplained hemorrhagic fever were received by Special Pathogens Branch, CDC for diagnostic assessment (Table 1). The shipment also contained sera from nine close contacts, mainly household members. A second shipment, which arrived on September 20, 2000, contained acute-phase sera from an additional 15 hospitalized patients and 12 contacts. Subsequent specimens from Saudi Arabia and Yemen were submitted for confirmation and more detailed analysis. All work with potentially infectious material was performed in a biosafety level 4 maximum containment facility.

Virus Antigen, IgM, and IgG Detection in Patient Sera

Patient sera were tested for the presence of RVFV or Crimean-Congo hemorrhagic fever virus (C-CHFV) antigen, or immunoglobulin (Ig) M or IgG antibodies reactive with these viruses and Alkhurma virus, a member of the tick-borne encephalitis (TBE) complex that was recently discovered in Saudi Arabia (4). RVFV and C-CHFV antigen-capture assays were performed in an enzyme-linked immunosorbent assay (ELISA) format essentially as described (5). The RVFV assay

Table 1. Results of diagnostic testing of initial Saudi Arabian Rift Valley fever outbreak specimens[a]

		ALK		C-CHFV			RVFV				
Patient ID[b]	Category[c]	IgM	IgG	Ag	IgM	IgG	Ag	IgM	IgG	ISOL	PCR
10901	Suspected case-patient	-	-	-	-	-	POS	-	-	POS	POS
10902	Suspected case-patient	-	-	-	-	-	POS	-	-	POS	POS
10904	Suspected case-patient	-	-	-	-	-	-	POS	POS	POS	POS
10905	Suspected case-patient	-	-	-	-	-	POS	-	-	POS	POS
10906	Contact	-	-	-	-	-	-	-	-	-	-
10907	Contact	-	-	-	-	-	-	-	-	-	-
10908	Contact	-	-	-	-	-	-	-	-	-	-
10909	Contact	-	-	-	-	-	-	-	-	-	-
10910	Contact	-	-	-	-	-	-	-	-	-	-
10911	Contact	-	-	-	-	-	-	-	-	POS	POS
10912	Contact	-	-	-	-	-	-	POS	-	-	-
10913	Contact	-	-	-	-	-	-	-	-	-	-
10914	Contact	-	-	-	-	-	-	-	-	-	-
10931	Suspected case-patient	-	-	-	-	-	POS	-	-	POS	POS
10933	Suspected case-patient	-	-	-	-	-	-	POS	POS	-	-
10935	Suspected case-patient	-	-	-	-	-	-	-	-	-	-
10937	Suspected case-patient	-	-	-	-	-	POS	POS	-	POS	POS
10939	Suspected case-patient	-	-	-	-	-	-	POS	-	-	-
10941	Suspected case-patient	-	-	-	-	-	-	-	-	-	-
10943	Suspected case-patient	-	-	-	-	-	POS	-	-	POS	POS
10945	Suspected case-patient	-	-	-	-	-	-	POS	-	POS	POS
10947	Suspected case-patient	-	-	-	-	-	POS	POS	-	POS	POS
10949	Suspected case-patient	-	-	-	-	-	-	POS	-	POS	POS
10951	Suspected case-patient	-	-	-	-	-	POS	-	-	POS	POS
10953	Suspected case-patient	-	-	-	-	-	POS	-	-	POS	POS
10955	Suspected case-patient	-	-	-	-	-	POS	POS	-	POS	-
10957	Suspected case-patient	-	-	-	-	-	POS	-	-	POS	POS
10959	Suspected case-patient	-	-	-	-	-	POS	-	-	POS	POS
10960	Contact	-	-	-	-	-	-	-	-	-	-
10961	Contact	-	-	-	-	-	-	-	-	-	-
10962	Contact	-	-	-	-	-	-	POS	-	-	-
10963	Contact	-	-	-	-	-	-	POS	-	-	-
10964	Contact	-	-	-	-	-	-	POS	POS	-	-
10965	Contact	-	-	-	-	-	-	-	-	-	-
10966	Contact	-	-	-	-	-	-	-	-	-	-
10967	Contact	-	-	-	-	-	-	-	-	-	-
10968	Contact	-	-	-	-	-	-	-	-	-	-
10969	Contact	-	-	-	-	-	-	-	-	-	-
10970	Contact	-	-	-	-	-	-	POS	-	-	-
10971	Contact	-	-	-	-	-	-	-	-	-	-

[a]RVFV, Rift Valley fever virus; ALK, Alkhurma virus; Ig, immunoglobulin; C-CHFV, Crimean-Congo hemorrhagic fever virus; ISOL, virus isolation; PCR, polymerase chain reaction; POS, positive results; -, negative results.
[b]Initial specimens were all from persons living in Jizan Province.
[c]Suspected case-patients were defined as described earlier (3).

used polyclonal hyperimmune ascitic fluid raised against RVFV strain Zagazig 501 as the capture antibody and rabbit hyperimmune serum raised against RVFV Zagazig 501 as the detector antibody. The C-CHFV assay used a sheep hyperimmune serum raised against a South African C-CHFV strain as the detector antibody, and a mouse hyperimmune ascitic fluid raised against C-CHFV strain IbAr10200 as the capture antibodies (6). IgM antibody titers were determined by IgM antibody–capture ELISA, with RVFV, C-CHFV, or Alkhurma virus–infected cell slurry prepared as described (5). IgG antibody titers were determined by using RVFV, C-CHFV, and Alkhurma virus–infected cell antigens in an ELISA format similar as to that described previously (5).

Virus Isolation and RNA Extraction

Viral RNA was obtained directly from patient blood or serum collected during the outbreak or from virus isolated from patient serum that was passaged once in Vero E6 cells. A virus stock was prepared by placing 100 µL of patient serum (200010901) onto a confluent monolayer of Vero E6 cells in a T-25 flask. After the virus was allowed to absorb for 1 h at 37°C, 6–7 mL of Dulbecco, modified Eagle medium supplemented with 5% fetal calf serum (FCS) and antibiotics, was added to the T-25 flask and allowed to incubate at 37°C, 5% CO_2. Cell cultures were checked daily for cytopathic effect (CPE), and after approximately 75% CPE was observed, remaining cells were scraped off and combined with the supernatant. A low-speed centrifugation removed most debris, and the resulting supernatant was stored at -80°C. Some cells were retained to perform immunofluorescence (IFA) directed at RVFV to check for positive cultures. Two hundred microliters of passage 1 cell/supernatant was placed into 1 mL of TriPure (Roche, Indianapolis, IN) for RNA purification. Saudi Arabia sample 2003043 and Yemen sample 2001373 were prepared by placing 200 µL of blood or serum, respectively, directly into 1 mL of TriPure. RNA was extracted onto glass beads by using a RNAid kit (Bio101, Carlsbad, CA) according to a modified protocol (7).

Indirect Immunofluorescence Assay and RT-PCR

Virus-infected cells were tested for RVFV antigens by indirect immunofluorescence assay essentially as described (5), except cells were incubated with anti-RVFV immune mouse ascitic fluid.

The nucleic acid sequences of the partial S, M, and L segments of RVFVs were amplified by using a one-step reverse transcriptase polymerase chain reaction (RT-PCR) (Promega Access kit, Madison, WI), according to manufacturer's protocol. The primers NSn (5′- TATCATGGATTACTTTCC-3′) and NSc (5′- CCTTAACCTCTAATCAAC-3′) were used to amplify a 661-Nt region (excluding primer sequences) of the virus S segment region encoding the NSs protein (8). The primers RVFFORI (5′-GTCTTGCTTGAAAAGGGAAAA-3′) and RVFREVE (5′-CCTGACCCATTAGCATG-3′) were used to amplify a 708-Nt region (excluding primers) of the virus M segment region encoding the G2 protein. Primers Wag (5'-ATTCTTATTCCCGAATAT-3′) and Xg (5′-TTGTTTTGC-CTATCCTAC-3′) were used to amplify a 176-Nt (excluding primers) region of the L segment (9). The primers RVFREVE together with primer RVFFORA (5′-TGCTACCAGACT-CATTTGTC-3′) were used to amplify the initial diagnostic fragment of 186 Nt (excluding primers) of the virus M RNA genome segment region encoding the G2 protein.

Electrophoresis of amplified DNA products was done on a 1.7% agarose gel in Tris-acetate-EDTA buffer. Following staining with ethidium bromide, specific DNA bands were located by UV translumination, sliced from the gel, and purified by using Qiaquick spin columns (Qiagen, Valencia, CA). Dye terminator cycle sequencing reactions were performed by using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kits with AmpliTaq DNA Polymerase FS (Applied Biosystems, Foster City, CA). Reaction products were purified by using Centri-sep spin columns (Princeton Separations, Adelphia, NJ) and sequences determined with an ABI 377 automated DNA sequencer (Applied Biosystems). Output chromatograms were analyzed with Sequencher 3.0 software (Gene Codes Corp., Ann Arbor, MI). RVFV sequences were aligned with those of previously characterized RVFVs (10) by using the PILEUP program of the Wisconsin Package Version 10.2 (Genetics Computer Group, Inc., Madison, WI). Maximum likelihood phylogenetic analysis was carried out by using PAUP4.0b10 (Sinauer Associates Inc., Sunderland, MA).

Results

Initial RVF Diagnosis

In early September 2000, the Ministry of Health of Saudi Arabia received reports of unexplained hemorrhagic fever cases in the southern Tehama (coastal plain) region of southwestern Saudi Arabia. Subsequently, reports were also obtained by the Yemen Ministry of Health of a similar disease in the adjoining Tehama region of Western Yemen. Initial specimens included acute-phase sera from four hospitalized patients with suspected cases and sera from nine contacts (mostly family members). Based on the available clinical information, the differential diagnostic included Rift Valley fever, Crimean-Congo hemorrhagic fever, and Tick-borne encephalitis-like viruses. The four serum samples from the suspected case-patients were tested by antigen-capture ELISA with RVFV- or C-CHFV-reactive antibodies; IgM-capture ELISA with RVFV, C-CHFV, or Alkhurma virus–infected cell lysate antigens; IgG ELISA with RVFV, C-CHVF, or Alkhurma virus–infected cell slurry antigen; virus isolation with Vero E6 cells; and RT-PCR assays for detection of RVFV, C-CHFV, or TBE-complex virus RNA. Evidence of RVFV infection was found in all four patients with suspected cases (Table 1). No evidence of C-CHFV or Alkhurma virus infection was found. Three of four acute-phase sera were positive by RVFV antigen-capture ELISA, and the single negative serum was positive for RVFV IgM and IgG, suggesting a later

stage of infection in this case. All four sera were positive by RVFV RT-PCR assay and subsequently yielded infectious RVFV by culture on Vero E6 cells.

Of the nine sera from close contacts, two showed evidence of RVFV infection. One was RT-PCR positive and virus isolation positive, and the other was positive for RVFV IgM antibodies. A second shipment of specimens yielded similar results, again confirming that RVFV was responsible for the outbreak in Saudi Arabia. In this second shipment, 13 of 15 hospitalized suspected case-patients had evidence of RVFV infection. Four contacts of case-patients also showed evidence of RVFV infection.

The rapid RVFV RT-PCR assay appeared to be a useful complement to the RVFV antigen and IgM-capture ELISA tests for diagnosis of acute illness, as it detected virus RNA in 15 of 16 serum samples that were subsequently found to be RVFV positive. Overall correlation between the various RVFV diagnostic assays was good. Nucleotide sequence analysis of the 186-Nt (excluding primer regions) PCR products amplified from these initial specimens confirmed the virus identity as RVFV and showed no nucleotide differences between the viruses detected in these Saudi Arabian patients. In addition, no nucleotide differences were detected in this 186-Nt region relative to viruses detected in an earlier outbreak in East Africa in 1997 (data not shown) (11).

Detailed Genetic Analysis

RNA extracted from three representative viruses was chosen for more detailed genetic analysis. These included RNA from RVFV isolate (strain 200010901) obtained from the first RVFV-infected case-patient to be laboratory confirmed and representing the early phase of the outbreak in Saudi Arabia. This isolate was obtained from a serum sample collected on September 13, 2000, from this case-patient (Table 1), who was infected in Jizan Province. RNA extracted from a serum sample collected late in the outbreak in Saudi Arabia was also included (Table 2). This serum sample was collected on November 22, 2000, from a case-patient infected in Asir Province. The third RNA sample was extracted from a blood sample obtained from a case-patient in Yemen. With these RNA samples, we hoped to detect any genetic variation in the RVFVs active during the early and late phases of the outbreak in Saudi Arabia and to evaluate whether the same virus strain was responsible for disease in Saudi Arabia and Yemen.

A single nucleotide difference was observed between each of the Saudi Arabia and Yemen virus S RNA genome segment fragments analyzed (601 nt). Similarly, no nucleotide differences were found between the Saudi Arabia 200010901 and Yemen 2003043 virus M RNA genome segment fragments we analyzed (510 nt), and these differed from the Saudi Arabia 2001373 virus fragment by only 1 nt. All three viruses were identical for the L RNA genome segment fragment we analyzed (129 nt). These data demonstrate that the viruses in the early and late stages of the RVF outbreak in Saudi Arabia are virtually identical to one another and to the virus causing disease in Yemen.

The results of phylogenetic analysis of the nucleotide sequence differences among the S, M, and L RNA genome fragments of the Saudi Arabia and Yemen viruses and previously described RVFVs are shown (Figure). Earlier maximum likelihood analyses had separated RVFVs into three broad groups, which predominantly contained viruses from North Africa, West Africa, and East/Central Africa (10). All three RNA segment trees obtained here have the Saudi Arabia and Yemen viruses grouped with the East/Central Africa viruses. Specifically, the S, M, and L RNA genome segments of the Saudi Arabia and Yemen viruses are closely related to those of viruses previously detected in outbreaks in East Africa, as represented by the Kenya 1997 and Madagascar 1991 virus isolates (Figure, A, B, and C). The nucleotide changes in the S, M, and L RNA genome segment fragments observed among the closely related Saudi Arabia/Yemen viruses and the Kenya 1997 and Madagascar 1991 viruses are synonymous changes, resulting in no amino acid differences among these viruses.

Discussion

Using a combination of RVFV IgM and antigen-capture ELISA tests, along with the RT-PCR assay, we quickly identified RVFV as the cause of a large outbreak in Saudi Arabia reported in September 2000. The RT-PCR assay proved to be an excellent complement to the antigen and antibody ELISA detection systems for the initial rapid diagnosis of RVF. Virus-specific antibodies were present in three of four specimens that were positive by both virus isolation and PCR but negative by antigen capture, suggesting that immune complex formation (antibody blocking of antigen) may be the basis for the lower sensitivity of the antigen-capture assay. Although the IgM assay failed to identify 9 of 17 laboratory-confirmed (by virus isolation or PCR) acute RVF cases, the assay did detect recent RVFV infections in five contacts (mostly close family members) and one acute case that would have been missed on the basis of virus isolation or PCR assay only. The data from this study and others (12) demonstrate the importance of combining assays for the detection of virus (antigen capture, RT-PCR,

Table 2. Specimens chosen for more detailed virus genetic analysis, Saudi Arabia and Yemen

No.	Collection date	Location	Specimen type	Passage history[a]
200010901	Sept. 13, 2000	Jizan Province, Saudi Arabia	Virus isolate	P1
2001373	Nov. 22, 2000	Asir Province, Saudi Arabia	Serum (human)	NA
2003043	Oct. 28, 2000	Northwest Yemen	Blood (human)	NA

[a]P1, first passage; NA, not applicable.

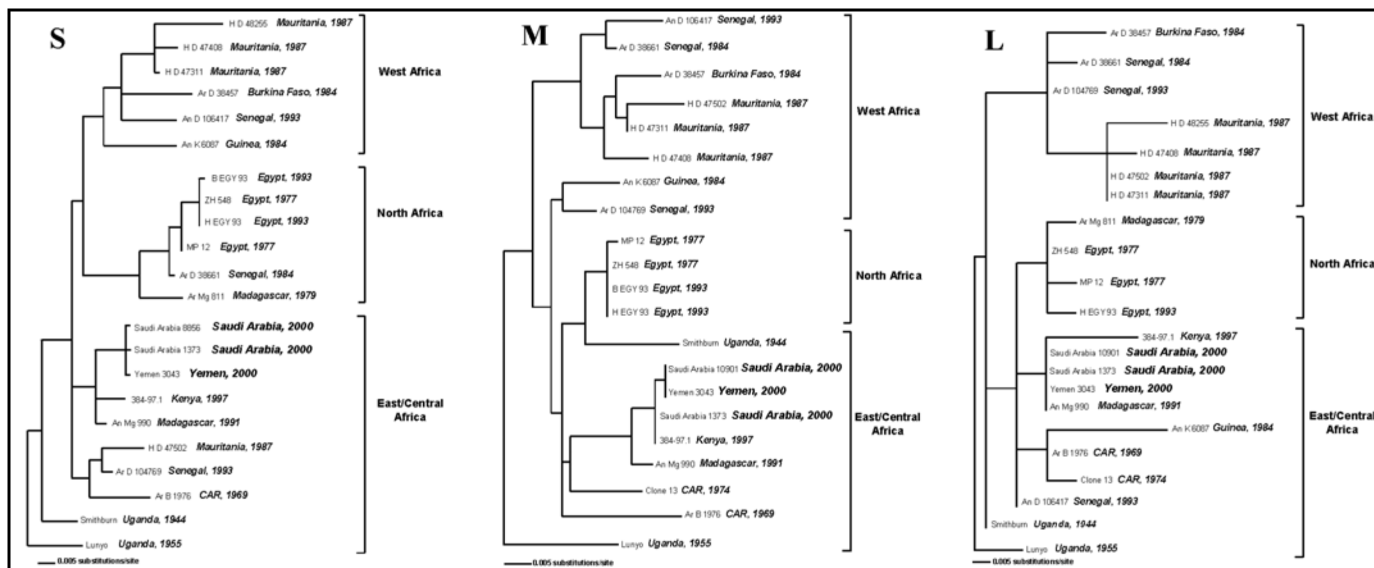

Figure. Phylogenetic relationship of the S, M, and L RNA segments of Rift Valley fever viruses. Maximum likelihood analysis of the nucleotide (nt) sequence differences among a 661-nt region of S RNA segment (Panel A), a 708-nt region of the M RNA segment (Panel B), and a 176-nt region of the L RNA segment (Panel C) of RVF viruses was performed by using PAUP4.0b10 (Sinauer Associates Inc., Sunderland, MA).

or virus isolation) and the detection of virus-specific IgM to ensure that no acutely ill RVFV patients are missed.

This RVF outbreak, the first confirmed outside Africa, illustrates the potential for this disease to spread to other regions of the world. Virus activity on the Arabian Peninsula resulted in a considerable amount of disease activity from September 2000 to February 2001 (3,13). In Saudi Arabia, as of February 2001, 884 seriously ill, hospitalized RVF patients were identified, with 124 deaths. In Yemen, 1,087 cases were estimated to have occurred, with 121 deaths (14). The outbreak involved a broad geographic area, including Jizan and Asir Provinces in southwestern Saudi Arabia and much of the western coastal plain of Yemen. Because of the magnitude of this outbreak and the large geographic area it encompassed, the total number of human RVFV infections remains unknown. Data from previous outbreaks suggest that the number of hospitalized RVF patients identified during a large outbreak represents only a small percentage (<1%) of the total number of infections (2). Our finding of six laboratory-confirmed RVFV infections among household contacts is consistent with the view that hospitalized patients represent a small fraction of the number of infected persons. Based on these and earlier observations, the number of human infections during this epidemic must have been considerable. Large numbers of livestock were also affected, causing substantial losses and economic impact in the rural areas hardest hit by the disease. Further impact of the outbreak included trade and travel restrictions.

Genetic analysis of S, M, and L segments of the viruses detected in Saudi Arabia and Yemen indicated that essentially the same virus caused both outbreaks. Few genetic differences were detected between viruses sampled early and late in the outbreak or from the distant geographic regions of Jizan and Asir Provinces in Saudi Arabia and areas of Western Yemen. The lack of substantial genetic variation in these viruses, together with the lack of earlier disease reports, suggests that RVFV has only recently been introduced onto the Arabian Peninsula.

Phylogenetic comparison of the nucleotide sequence differences between the Arabian Peninsula RVFV S, M, and L segments and those of previously characterized RVFV isolates showed a close relationship between the Saudi Arabia/Yemen RVFVs and those circulating earlier in East Africa, particularly with the viruses responsible for the large RVF outbreak seen in the region in 1997–98 (11). These results are consistent with the introduction of RVFV into Saudi Arabia and Yemen from East Africa. While genetic reassortment has been observed in RVFVs associated with outbreaks in various geographic regions of Africa (10), the close phylogenetic relationship of the S, M, and L RNA segments of the 2000–01 Saudi Arabia and Yemen viruses and the earlier 1997 and 1991 Kenya and Madagascar viruses, respectively, provided no evidence of genetic reassortment among these viruses.

The mechanism of introduction of the virus into the Saudi Arabia and Yemen remains unknown. However, commercial trade of livestock is active from East Africa to the Arabian Peninsula, and disease is known to be endemic in the East African region. While no hospitalized RVF patients have been reported in 2002, whether this RVF lineage has become established in the Arabian Peninsula remains unclear. Surveillance of humans, livestock, and vector populations will continue to address this question.

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Co-feeding Transmission and Its Contribution to the Perpetuation of the Lyme Disease Spirochete *Borrelia afzelii*

Dania Richter,* Rainer Allgöwer,* and Franz-Rainer Matuschka*

To determine whether direct passage of spirochetes between co-feeding vector ticks contributes to the likelihood that the Lyme disease spirochete *Borrelia afzelii* will perpetuate in nature, we compared the effects of time and space on transmission efficiency between simultaneously feeding ticks. The likelihood of co-feeding transmission increases with duration of attachment of the infecting tick. Co-feeding transmission becomes less efficient as distance from the infecting tick increases. Approximately 6 times as many ticks acquire infection when feeding on infected mice than when co-feeding with infected ticks. Both subadult stages of the wood tick *Ixodes ricinus* infrequently co-infest mice and voles in nature; on approximately 1 in 20 small rodents, larvae co-feed with spirochete-infected nymphs. Because only 1 in 100 larvae in nature appear to acquire spirochetal infection when co-feeding with infected nymphs, perpetuation of *B. afzelii* depends largely on horizontal transmission of such pathogens from previously infected mice to noninfected larvae.

Risk of Lyme disease generally is associated with the presence of ticks of the *Ixodes ricinus* complex and with particular rodents that support dense spirochetal infections. Lyme disease spirochetes migrate through the skin of their vertebrate hosts, where they are imbibed by feeding vector ticks; infectivity increases as the spirochetes multiply and disseminate. Rodent hosts are most infectious to ticks approximately 2 weeks after they have acquired infection; the hosts then infect virtually all ticks feeding on them (1,2). These pathogens may also pass directly from infected to noninfected ticks while the ticks are feeding simultaneously in close proximity and before the spirochetes have disseminated throughout the skin of their hosts (3–5). *Thogotovirus,* another tick-borne agent, can pass directly from infectious to noninfected ticks, even in a nonviremic host (6). This direct tick-to-tick mode of transmission may be crucial in perpetuating tick-borne encephalitis virus because ticks could acquire infection when feeding on immune hosts (7). Even vertebrate hosts without a systemic infection might, thereby, infect vector ticks (6).

Time and space may limit the efficiency of spirochetal transmission between co-feeding ticks. Although Lyme disease spirochetes appear to be cotransmitted efficiently when noninfected and infected ticks feed simultaneously on the same ear or other body part of a mouse, no larvae become infected when attached to the opposite ear or to the animal's back (3–5). Only a few noninfected ticks become infected when permitted to attach at random to an animal that is serving simultaneously as host to spirochete-infected ticks, even when numerous infected ticks have been applied (4,5). A temporal effect may similarly limit the efficiency of co-feeding transmission because spirochetes are deposited in the skin only after the infecting tick has been feeding for approximately 2 days (8), and such ticks detach 1 or 2 days later. In the event of disseminated infection in the tick, transmission would occur somewhat more rapidly. The combined role of time and distance in the efficiency of co-feeding transmission, however, has not been rigorously examined.

Direct passage of spirochetes between co-feeding vector ticks may contribute to the likelihood that Lyme disease spirochetes will perpetuate in nature. To examine this hypothesis, we evaluated the effects of interfeeding distance and time interval between infected and noninfected ticks on the efficiency of co-feeding transmission. In particular, we compared the effects of time and space on transmission efficiency between simultaneously feeding ticks. In addition, we determined the frequency of infection in ticks randomly feeding on mice that were simultaneously parasitized by an infectious tick and compared that estimate with the frequency of infection for ticks feeding on mice that had previously served as host to an infectious tick. Lastly, we estimated the frequency of larvae co-feeding with infected nymphal ticks on rodents in disease-endemic foci of transmission.

Materials and Methods

Outbred hairless mice (*Mus musculus*, SKH-1 strain), originally purchased from Charles River Deutschland (Sulzfeld, Germany), were bred and maintained in the laboratory. *I. ricinus* ticks were infected by *Borrelia afzelii* spirochetes.

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Virtually all such nymphal ticks contained spirochetes. The strain of *B. afzelii* originated from a naturally infected nymphal *I. ricinus* tick collected from vegetation in suburban Berlin (9). We have previously characterized this strain and maintained it by serial passage through jirds (*Meriones unguiculatus*) and ticks. Noninfected laboratory-reared larval *I. ricinus* were derived from adults in their third generation of continuous laboratory rearing by using noninfected hosts. To confirm that such larvae were free of spirochetes, pooled samples of each batch were routinely analyzed by polymerase chain reaction (PCR) amplification of a fragment of the 16S rRNA gene (see below).

In experiments designed to determine how readily spirochetes pass between co-feeding ticks, a single *B. afzelii*–infected nymph was permitted to attach between the shoulders of each mouse. Approximately 100 noninfected larvae were brushed onto each mouse to attach in close proximity to the feeding nymph and also at 1 and 2 cm ± 0.2 cm from the site of nymphal attachment. Any larvae that had attached elsewhere on the hairless mouse were promptly removed with forceps. Larval ticks were placed on hosts either at the time of nymphal attachment or at 24, 48, or 72 hours thereafter. Three mice were used for each time point. Each infested mouse was kept individually in a wire-mesh restraining tube suspended over water until the mouse was free of all ticks. Mice were fed standard laboratory chow and apple slices; the contents of the water pan were changed twice a day. When larvae were engorging rapidly and had become almost replete, generally approximately 48 hours after attachment, they were carefully removed with forceps and transferred to separate tubes according to their distance from the nymph. The tubes were half-filled with water-saturated plaster of paris. Engorged larvae were kept at 20±2°C under a light-dark regimen (16:8) until they molted. To confirm successful infection in each mouse, these hosts underwent xenodiagnosis with noninfected larval ticks at 2 weeks after the infected nymphal ticks had been permitted to feed. After engorged larvae had molted to the nymphal stage, at least 10 specimens from each group were examined for the presence of spirochetes by dark-field microscopy. If the apparent rate of infection in a group was ≤10%, the remaining ticks were analyzed by PCR.

For PCR, the body of a tick was opened, and the contained mass of soft tissue was dissected out in physiologic saline and transferred to a tube containing 180 µL lysis buffer (ATL Tissue Lysis Buffer, Qiagen, Hilden, Germany) and 20 µL proteinase K (600 mAU/mg). Midguts were lysed at 56°C overnight. DNA was extracted by using the QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions, eluted with 50 µL elution buffer, and stored at –20°C until PCR was performed.

DNA of Lyme disease spirochetes was detected by amplifying a 650-nucleotide segment of the gene encoding the 16S rRNA. To increase sensitivity for detecting spirochetal DNA in ticks, we used nested PCR. Aliquots of DNA suspensions (2 µL) were diluted to 50 µL by using 200 µm of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 0.5 U Taq polymerase (Qiagen) as well as 15 pmol of the outer primer pair and PCR buffer supplied with the Taq polymerase. We used the following primer sequences of the 16S rRNA gene (10): outer primers (5'–3') 16S1A – CTAACGCTGGCAGTGCGTCTTAAGC and 16S1B – AGCGTCAGTCTTGACCCAGAAGTTC (positions 36–757). The mixture was placed in a thermocycler (PTC 200, MJResearch, Biozym, Germany), heated for 1 min at 94°C, and subjected to 30 cycles, each including a 20-sec denaturation at 94°C, a 20-sec annealing reaction at 63°C, and a 40-sec extension at 72°C. A final extension for 2 min at 72°C was added to the last cycle. After the first amplification with the outer set of primers, 2 µL of the amplification product was transferred to a fresh tube containing 48 µL of the reaction mixture described above, except that 2.5 mM $MgCl_2$ and 20 pmol of the inner primer pair: (5'–3') 16S2A–AGTCAAACGGGATGTAGCAATAC and 16S2B – GGTATTCTTTCTGATATCAACAG (positions 66–720) were used. This mixture was subjected to 35 amplification cycles by using the cycle conditions described above, except that the annealing reaction was performed at 56°C and the extension reaction lasted 30 sec. DNA was extracted, reaction vials were prepared for amplification, templates were added, and products underwent electrophoresis in separate rooms. For comparison, each PCR amplification series included DNA from a laboratory-reared nymph that had fed in its larval stage on *B. burgdorferi* s.s.–infected jirds. In each fifth reaction mix, water was added instead of extracted DNA to serve as a negative control. PCR products were detected by electrophoresis in a 1.5% agarose gel stained with ethidium bromide.

To determine the frequency of subadult tick stages coinfesting rodents in nature, we captured small mammals in live traps (Longworth Scientific Instruments, Abingdon, U.K.) baited with apple, grain, and cotton. Rodents were captured in two sites in southwestern Germany (Kappel and Tübingen) and one in adjacent France (Petite Camargue Alsacienne) during April through October of 1993–1995. Each rodent was caged over water until all ticks had detached. The contents of the water pan were inspected twice daily, and ticks were promptly removed, counted, and identified. Rodents were released at the point of capture.

To determine the prevalence of Lyme disease spirochetes in questing nymphal ticks in these sites, such ticks were collected once a month during April through October of 1993–1995 by means of a flannel flag dragged through brushy vegetation. The ticks were confined in screened vials and stored at 10°C±1°C until they were identified as to stage and species and examined for spirochetes. Field-collected ticks were dissected and their midguts examined for spirochetal infection by dark-field microscopy.

Results

First, we determined whether the duration of nymphal attachment before larval attachment affects the likelihood of spirochetal transmission between co-feeding ticks. Few larvae

acquired infection unless they had attached some time after the infecting nymph had attached (Table 1). Some larvae became infected when they were placed on the host after 2 days; more larvae became infected when they began to feed on the 3rd day of nymphal attachment, when the infecting nymph was becoming replete and had begun to detach. The likelihood of co-feeding transmission therefore increases with duration of attachment of the infecting tick.

We then evaluated the effect of interfeeding distance on the efficiency of co-feeding transmission. About half of the larvae became infected when they fed virtually in contact with the infecting nymph and when the nymph had become replete (Table 1). Fewer than a quarter became infected when feeding 1 cm from the infecting tick and even fewer at 2 cm distance. These findings suggest that co-feeding transmission becomes less efficient as distance from the infecting tick increases.

The efficiency of transmission between ticks feeding randomly, but simultaneously, on the same host was compared to the frequency of infection in ticks feeding on mice that had been infected 2 weeks earlier. In the simultaneously feeding ticks, cohorts of noninfected larvae were permitted to attach to mice 3 days after one spirochete-infected nymph was permitted to attach, and just before it had become replete. Many fewer simultaneously feeding ticks than sequentially feeding ticks acquired infection in this experiment (Table 2). Approximately 6 times as many ticks acquired infection in the course of feeding on infected mice than when co-feeding with infected ticks.

The likelihood that larval and nymphal ticks might acquire spirochetal infection by co-feeding in nature was established by analyzing the distribution of subadult ticks on rodents captured in endemic foci of transmission and determining the prevalence of Lyme disease spirochetes in questing nymphs. Larvae, but no nymphs, were found on approximately two thirds of yellow-necked mice (*Apodemus flavicollis*), wood mice (*A. sylvaticus*), and bank voles (*Clethrionomys glareolus*) (Table 3). Nymphs and larvae coinfested about a fifth of mice and even fewer voles. Two thirds of garden dormice (*Eliomys quercinus*), however, were coinfested by larvae and by nymphs. Of the nymphal ticks questing in these sites, approximately one quarter (26.4%) were infected by Lyme disease spirochetes. In contrast to the relationship in garden dormice, both subadult stages of the wood tick infrequently coinfested mice and voles; in nature, larvae co-feed with spirochete-infected nymphs only on approximately 1 in 20 small rodents.

Discussion

The experimental demonstration that tick-borne pathogens could perpetuate in nature in the absence of reservoir hosts that develop systemic infections (6) transformed a previously central epidemiologic concept. Thereby, a reservoir host, used in the sense of the alternative to the vector host, need not support the dissemination of the pathogen. Even a virus-immune vertebrate host permits passage of that virus between simultaneously feeding vector ticks (7). The concept applies similarly to Lyme disease spirochetes transmitted by ticks feeding in close proximity to each other on competent rodent hosts (3–5) and on spirochete-incompetent sheep (11). At least in the laboratory, diverse pathogens can pass directly between vector ticks.

The efficiency of co-feeding transmission may depend on interfeeding distance. In the case of *Thogotovirus*, distance appears to make little difference. The virus readily passes between ticks that are feeding on guinea pigs as far as 160 mm apart, and these hosts remain nonviremic (12). Co-feeding transmission of tick-borne encephalitis virus, in contrast, is more efficient if ticks feed in close proximity than if separated by a distance of 1 cm. Langerhans cells appear to aid transmission (13). The ability of Lyme disease spirochetes to pass between co-feeding vector ticks is less pronounced. Although spirochetes readily pass between ticks confined in the same feeding chamber, none do so when the infected ticks feed on the back of a mouse while noninfected ticks are feeding on the mouse's head (3), when attached to different ears of a jird (5), or when the distance separating the infected from the noninfected ticks is 3 cm (4). We found that the critical distance between the co-feeding pair was approximately 1 cm. Although an occasional tick might become infected by *B.*

Table 1. Spirochetal infection in larval *Ixodes ricinus* ticks that fed on mice during the period of attachment of a single *Borrelia afzelii*–infected nymph and that fed at specified distances from the infecting nymph[a]

Duration of nymphal attachment before larvae attached (days)	Distance between nymph and larvae (cm)	Infection in co-feeding larvae	
		No. examined	% infected
0	Nil	68	0
	1	83	0
	2	51	0
1	Nil	125	1.6
	1	74	0
	2	124	0
2	Nil	67	29.9
	1	87	5.7
	2	54	1.9
3	Nil	94	55.3
	1	82	25.6
	2	160	6.3

[a]Each feeding sequence was replicated three times.

Table 2. Spirochetal infection in larval *Ixodes ricinus* ticks that fed randomly on bodies of mice beginning at 3 days and 14 days after a single *Borrelia afzelii*–infected nymph had begun to feed

Duration of nymphal attachment before larvae attached (days)	Infection in larvae	
	No. examined	% infected
3	88	13.6
14	82	85.4

Table 3. Proportion of captured rodent hosts infested by larval and nymphal *Ixodes ricinus* ticks, southwestern Germany and Alsace

Hosts		% hosts infested by			
Kind[a]	No.	None	Larvae alone	Nymphs alone	Larvae and nymphs
Af	215	12.1	65.1	2.3	20.5
As	128	14.1	62.5	0.8	22.7
Cg	183	25.1	60.1	0.5	14.2
Eq	66	6.1	27.3	1.5	65.2

[a]Yellow-necked mice (*Apodemus flavicollis* [Af]), wood mice (*A. sylvaticus* [As]), bank voles (*Clethrionomys glareolus* [Cg]), and garden dormice *(Eliomys quercinus* [Eq]).

afzelii over a distance of 2 cm, transmission efficiency falls precipitously as the distance between co-feeding ticks approaches 1 cm.

Transmission efficiency also has a temporal component. In contrast to virus particles, which are present in the salivary glands at the time of attachment (14), North American Lyme disease spirochetes as well as *B. afzelii* are injected into the skin of a vertebrate host only after the infecting tick has been attached for more than a day (8, unpub. data). Because nymphal *I. ricinus* remain attached for approximately 3 days and larvae 1 day less, the co-feeding window remains open only briefly. A larva could not ingest spirochetes if it attached at the same time as did the infected co-feeding nymph. This scenario conforms well to our observations. Other *Ixodes* vector ticks feed approximately 1 day longer than do subadult *I. ricinus*, a pattern that explains why some larvae, described in other studies, became infected when permitted to attach at the same time as the infecting nymph (4,5). The relationship between time and distance is particularly complex because of the ability of these spirochetes to move rapidly through skin (15). They disseminate through this matrix only after the infecting tick has become replete and has detached (1). Indeed, we found that the efficiency of co-feeding transmission correlates inversely with distance between the feeding pair. In general, less than 14% of randomly attached ticks acquire infection by co-feeding transmission.

The number of co-feeding larval and nymphal ticks appears to affect the efficiency of transmission of Lyme disease spirochetes. No spirochetes are transmitted between co-feeding ticks when natural densities of subadult *I. scapularis* ticks infest the white-footed mouse (*Peromyscus leucopus)* (16). In contrast, up to 5% of 200 larvae acquire *B. burgdorferi* s.s., when co-feeding with as many as 40 infected nymphal *I. scapularis* ticks on the North American reservoir rodent (16). In nature, however, this density of subadult ticks on murine hosts is unlikely (16). Even fewer infected nymphal ticks generally feed on the European reservoir rodents (17). Although efficiency of transmission of Lyme disease spirochetes increases with density of co-feeding ticks, such tick densities are extremely rare in nature.

A synthetic model has recently been employed to estimate the overall contribution of co-feeding transmission to the intrinsic rate of natural increase (R_0) of populations of Lyme disease spirochetes (18). This model is based on major assumed parameters that include 1) competence of the vector and reservoir hosts, combined with duration of infectivity, and 2) proportion of feeding ticks that acquire infection, combined with the effect of distance between co-feeding ticks. Although conventional "systemic" transmission would be far more important than nonsystemic tick-to-tick transmission in the case of Lyme disease spirochetes, these considerations suggest that "any host that feeds large numbers of ticks should now be considered a candidate as an amplifying host"(18). Reservoir-incompetent vertebrate hosts appear to contribute an important component to the force of transmission.

We found that 1 cm appears to be the critical distance separating infectious from susceptible ticks that inhibits transmission of *B. afzelii* between simultaneously feeding ticks. If one assumes that infected reservoir rodents remain infectious throughout their lives, some 85% of larval *Ixodes* ticks acquire infection from rodents when feeding "sequentially," i.e., on hosts that had previously been infected by nymphal ticks. In contrast, less than 14% of larvae do so when they feed "simultaneously" with infected nymphs. In nature, about 20% of small rodents carry both subadult stages (19 and current study), and spirochetes infect approximately 26% of these nymphs. By simple multiplication, then, less than 1% of vector ticks (14 x 20 x 26 = 0.73%) would acquire spirochetal infection during co-feeding, and even fewer would become infected by *B. afzelii*. This calculation corresponds closely to our observation of spirochetal infection in larval ticks that had attached, in nature, to hosts that do not support spirochetal infection (20). The previous theoretical estimate suggests that six times as many infected vector ticks derive from larvae that fed on spirochete-infected hosts than would result if they co-fed with infected ticks on noninfected hosts (18). Our combined experimental and field-derived evidence, however, indicates that the transmission efficiency between sequentially feeding ticks exceeds that between co-feeding ticks by a ratio of at least 100:1 (85/0.73=116). Perpetuation of the Lyme disease spirochete *B. afzelii*, therefore, would depend largely on persistent dissemination of these pathogens throughout the skin of the competent vertebrate hosts on which the vector ticks mainly feed.

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Dr. Richter is a research associate at the Charité Medical School, Humboldt-University of Berlin. Her research interests focus on the immunologic and molecular interface of the host-vector-pathogen relationship in the epizootiology of Lyme disease.

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Binary Cumulative Sums and Moving Averages in Nosocomial Infection Cluster Detection[1]

Samuel M. Brown,* James C. Benneyan,† Daniel A. Theobald,‡ Kenneth Sands,§ Matthew T. Hahn,‡ Gail A. Potter-Bynoe,¶ John M. Stelling,# Thomas F. O'Brien,#** and Donald A. Goldmann¶**

Clusters of nosocomial infection often occur undetected, at substantial cost to the medical system and individual patients. We evaluated binary cumulative sum (CUSUM) and moving average (MA) control charts for automated detection of nosocomial clusters. We selected two outbreaks with genotyped strains and used resistance as inputs to the control charts. We identified design parameters for the CUSUM and MA (window size, k, a, b, p_0, p_1) that detected both outbreaks, then calculated an associated positive predictive value (PPV) and time until detection (TUD) for sensitive charts. For CUSUM, optimal performance (high PPV, low TUD, fully sensitive) was for $0.1 \leq \alpha \leq 0.25$ and $0.2 \leq \beta \leq 0.25$, with $p_0 = 0.05$, with a mean TUD of 20 (range 8–43) isolates. Mean PPV was 96.5% (relaxed criteria) to 82.6% (strict criteria). MAs had a mean PPV of 88.5% (relaxed criteria) to 46.1% (strict criteria). CUSUM and MA may be useful techniques for automated surveillance of resistant infections.

Nosocomial infections afflict 2 to 5 million patients in the United States annually and contribute to approximately 88,000 deaths (1,2). These infections are the second most frequent adverse effect of hospitalization (3,4). In most instances such infections are isolated, though studies have reported that from 2% (5,6) to 20% (7) to 60% (8) occur in clusters. A minimal estimate of the epidemic nosocomial infection burden is thus 40,000 cases annually (2% of 2,000,000), while a maximal estimate is conceivably five times that figure or more.

Most hospitals in the United States will have at least one outbreak per year, and large referral hospitals may have several (9). Nosocomial infection clusters can be difficult to diagnose and detect (5), which can have serious ramifications (10). Although options for computerized surveillance are increasing (11–15), many current methods for outbreak detection are effective only when substantial time has elapsed from the actual events. Techniques are often poorly automated (16–18), and few sophisticated cluster detection techniques have been employed in nosocomial infection surveillance (19–21).

Cumulative sums (CUSUMs) are statistical tools, based on a type of sequential hypothesis test, that were originally used in manufacturing processes to monitor production defect rates (22–24). Increments are added or decrements are subtracted from a running total over time, according to measurements of quality of serial items. The behavior of this cumulative sum is tracked until one of two conditions is met, with CUSUM values beyond these thresholds signaling either 1) a statistically significant change in quality to some prespecified level or 2) acceptance of the hypothesis of no change. CUSUMs have been used for several decades in health care settings, including for tracking operator improvements in performing procedure (25–27), monitoring fever curves in neutropenic patients (28), and detecting community *Salmonella* outbreaks (15). Several forms exist, including a so-called binary or Bernoulli CUSUM in which failure is rated as 1 and success as 0, a coefficient is subtracted, and the resulting values are added to the CUSUM. This binary form has not to our knowledge been applied to outbreak detection.

Moving averages (MAs) are in wide use in several fields, such as economics, where methods sensitive to sudden changes and filtering out background noise are required. Thus, for instance, economic indicators may be analyzed, with a MA calculated for the most recent values and compared with the historical mean for that indicator. An MA much higher than the historical mean indicates a statistical increase. MAs also are used in manufacturing quality control for the same reason (28). Although various MA techniques have been applied to disease rates in public health surveillance (29), they have not previously been applied to monitor changes in strain characteristics, such as antimicrobial resistance.

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We hypothesized that by treating antimicrobial resistance as the quality indicator of individual isolates, these techniques could be used to detect nosocomial clusters. Both techniques have been demonstrated in the quality control literature to be more sensitive to small rate changes than conventional p-type charts (22–24,30). We evaluated the performance of these techniques in simulated real-time detection of two genotypically characterized outbreaks of nosocomial infection caused by antimicrobial-resistant bacteria.

Methods

Outbreaks Investigated

The study hospital is a 330-bed tertiary-care pediatric facility in the northeastern United States. We selected all investigated nosocomial outbreaks of antibiotic-resistant bacteria in the study hospital for which genotyping data were available for the period 1995–2000, inclusive. An outbreak with genotyped organisms from 1997 was excluded because the causative agent, *Pseudomonas aeruginosa,* was sensitive to all standard therapeutic agents. This cluster was thus not a candidate for detection with our techniques. A line listing of all patients, with isolates, from both outbreaks is presented in the Table.

The Institutional Review Board of the study hospital authorized us to perform this study without obtaining informed consent. All patient identifiers were either deleted or irreversibly encrypted to ensure confidentiality.

Outbreak 1

An outbreak of surgical site infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) occurred in August through September 1999 in patients after cardiac surgery. Approximately 800 such surgeries are performed annually in the study hospital. Immediately after surgery, patients are cared for in the cardiovascular intensive care unit (CICU), which has 23 beds, 1,550 admissions per year, and an average length of stay of 4.4 days. After they are stabilized, the patients are transferred to the cardiac surgery ward (28 beds, >2,300 admissions per year; and average length of stay, 3 days). A single genotype of MRSA was isolated from four patients with evidence of deep/organ-space surgical infection after cardiac surgery. One of the genotypically identical isolates (O3-2) was detected by admission screening culture at another hospital to which the patient had been transferred. Another isolate (O3-7) was detected in a blood culture obtained at the hospital to which the patient had been transferred. Two surgical patients without clinical infection were colonized with isolates of a second genotype. Methicillin resistance was defined as a MIC of oxacillin of >0.5 mg/ml. All isolates of *Staphylococcus aureus* from any body site from the CICU and cardiac surgical ward were included in the analyses.

Outbreak 2

An outbreak of vancomycin-resistant enterococcus (VRE) occurred in May through June 2000 involving two units: the

Table. Cluster patients with isolates, dates, and sensitivities[a]

Patient	Culture date	Body site	PFGE type	Resistance phenotype[b]
MRSA				
O1-1	1/22/99	wd,bl	E	cli ERY tcy van SAM FEP OXA sxt CZO AMC amk
O1-2	7/10/99	no,ax	D	CLI ERY TCY van OXA sxt AMK
O1-3	7/10/99	sp	D	CLI ERY TCY van SAM FEP OXA sxt CZO AMC AMK
O1-4	8/23/99	wd	C	CLI ERY TCY van SAM FEP OXA sxt CZO AMC AMK
O1-5	9/3/99	wd	C	CLI ERY TCY van SAM FEP OXA sxt CZO AMC AMK
O1-6	9/6/99	wd	C	CLI ERY TCY van SAM FEP OXA sxt CZO AMC AMK
O1-7	9/13/99	bl	C	CLI ERY TCY van OXA sxt AMK
VRE				
O2-1	1/20/00	bl	non-B	VAN amc AMP
O2-2	5/12/00	st	B	VAN amc AMP
O2-3	5/14/00	fl	B	VAN amc AMP
O2-4	5/18/00	st	B	VAN amc AMP
O2-5	5/19/00	ti,st	B	VAN amc AMP TCY chl IPM nit
O2-6	5/24/00	st	B	VAN amc AMP
O2-7	6/23/00	fl	non-B	VAN AMC AMP

[a]MRSA, methicillin-resistant *Staphylococcus aureus*; PFGE, pulsed-field gel electrophoresis; O1, outbreak 1; O2, outbreak 2; VRE, vancomycin-resistant enteroccocus; bl, blood; sp, sputum; st, stool; wd, wound; ti, tissue; ax, axilla; no, nose; fl, fluid.

[b]Antibiotic codes in capital letters are resistant results; those in lowercase letters are susceptible: AMC, amoxicillin/clavulanate; AMP, ampicillin; AMK, amikacin; CLI, clindamycin; CZO, cefazolin; ERY, erythromycin; FEP, cefepime; OXA, oxacillin; SAM, ampicillin/sulbactam; SXT, trimethoprim/sulfamethoxazole; TCY, tetracycline; VAN, vancomycin.

bone marrow transplant unit and the general pediatric intensive care unit PICU. The bone marrow transplant unit is a 13-bed unit providing hematopoietic stem-cell transplantation. It has approximately 260 admissions per year, with an average length of stay of 12.9 days. When patients require ICU care, they are transferred to specially ventilated rooms in the PICU. The PICU is an 18-bed multidisciplinary unit, with approximately 1,650 admissions per year, and an average length of stay of 3.2 days. In May 2000, a patient colonized with VRE in the bone marrow transplant unit was transferred to the PICU. Other cases of VRE colonization or infection were detected in both the bone marrow transplant unit (4 cases) and the PICU (3 cases). Isolates of *Enteroccocus faecium* from five patients were demonstrated to be genotypically identical. Vancomycin resistance was defined as a MIC of vancomycin of ≥16 µg/mL. All isolates of *E.faecium* or unspeciated *Enterococcus* from any body site on the affected units were included in the analyses. Genotyping was performed by ARUP Laboratories (Salt Lake City, UT). Genotypic identity was defined according to a published procedure (31).

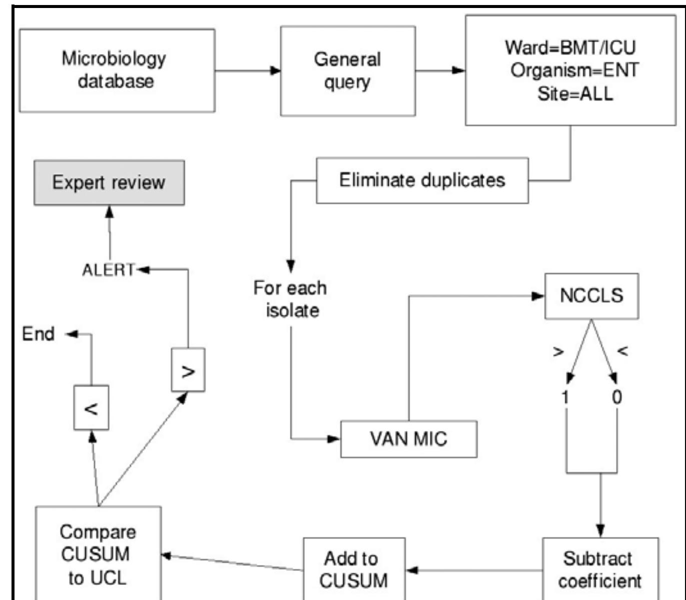

Figure 1. Data processing methodology for cumulative sums. BMT, bone marrow transplant unit; ICU, intensive care unit; ENT, enterococcus; VAN MIC, vancomycin minimum inhibitory concentration; NCCLS, National Committee for Clinical Laboratory Standards antibiotic susceptibility breakpoint; CUSUM, cumulative sum; UCL, upper confidence limit.

Data Acquisition:

Records for all inpatient cultures were downloaded from the study hospital's information system for January 1995–September 2000 into WHONET 5.0 (WHO Collaborating Center, Boston, MA). Species identification had been performed per standard laboratory procedures. Antibiotic sensitivities had been performed by measurement MIC with a MicroScan Walkaway-96 (Dade Behring, Inc., Deerfield, IL). Standard Kirby-Bauer technique was used when an organism failed to grow sufficiently to perform MIC analysis. Only final susceptibility readings were included. Susceptibility cutoffs were defined according to National Committee for Clinical Laboratory Standards (32). Indication for culture was specified as either clinical (C), routine surveillance (R), or outbreak investigation (O). Clinical cultures were ordered by treating physicians for care of the individual patient. Routine surveillance cultures included weekly stool screens for VRE and sentinel event screens. Infection control policy at the study hospital was to screen a high-risk unit (ICU or bone marrow transplant unit) if a patient was found to have new MRSA or VRE colonization or infection. Outbreak investigation cultures were those taken as part of a formal or informal outbreak investigation. Culture indications were determined from infection control records.

Data Analysis

Isolates of the same species from a given patient within 60 days of the previous isolate were excluded as duplicate isolates. All isolates of *E. faecium*, enterococcus, and *S. aureus* from the affected units were parsed by the BugCruncher program (Vecna Technologies, Hyattsville, MD) in the manner depicted in Figures 1 and 2. The resistance value (for binary tests 0 = susceptible or 1 = nonsusceptible; for quantitative tests, the actual MIC) for each isolate was then passed to CUSUM (binary only) or MA (binary and quantitative) modules, where alerts were generated on the basis of control limits. Test statistics and control limits were recalculated with the addition of each new isolate and processed in chronological order.

Each type of chart is calculated based on several design parameters (*w* and *k* for MA; a, b, p_0, p_1 for CUSUM). To explore performance robustness under various conditions, we selected a reasonable range of values for the control parameters for CUSUM ($0.01 \leq \alpha \leq 0.25$; $0.01 \leq \beta \leq 0.25$; $0.01 \leq p_0$

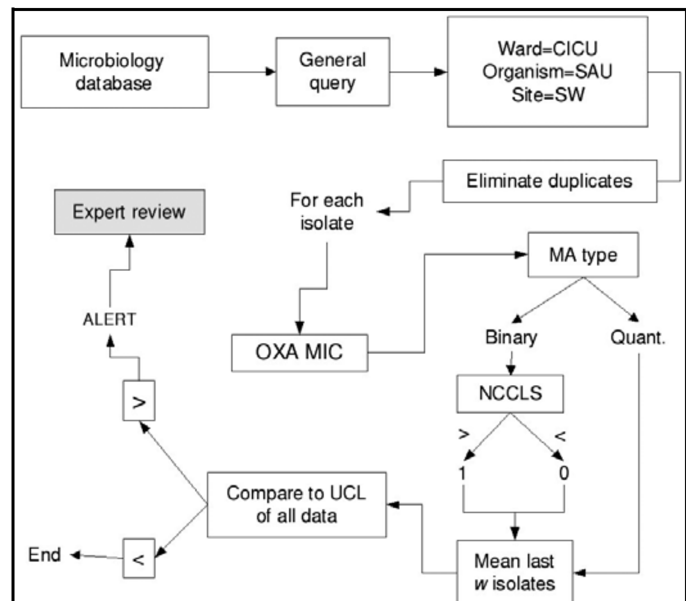

Figure 2. Data-processing methodology for moving averages. CICU, cardiac intensive care unit; SAU, *S. aureus*; SW, surgical wound; OXA MIC, oxacillin minimum inhibitory concentration; MA, moving average chart; NCCLS, National Committee for Clinical Laboratory Standards, antibiotic susceptibility breakpoint; UCL, upper control limit.

≤0.25; 0.01 ≤ p_1 ≤0.25) and MA (5 ≤ w ≤90 and 1 ≤ k ≤4) charts. Positive predictive value (PPV) was calculated for those design parameter values that detected both outbreaks. Further detail on these statistical methods and the formulae used for calculating their test statistics and detection thresholds are presented in the Appendix.

To validate the empirically derived design parameters in terms of theoretic performance, we then calculated the out-of-control (an actual change in incidence) and in-control (no change in incidence) time until detection (TUD) for the sets of design parameters that detected both outbreaks. We used standard methods for calculating TUDs, employing a Monte Carlo simulation program we wrote for that purpose. Simulations were run over 10,000 iterations.

Two definitions of cluster detection were used: generation of an alert at the second outbreak isolate (isolate-level detection) or during the first month of the outbreak (month-level detection). Positive predictive value (percent of detected events considered relevant) was calculated in the following manner (33) all detected events previously unnoted by infection control personnel were evaluated independently by two hospital epidemiologists (KS, DG). The epidemiologists classified each event as A) initiate investigation, B) monitor situation, or C) ignore. A "C" rating from both epidemiologists or a "B" from one and a "C" from the other was considered a false-positive result. True positives were divided into positives by strict criteria (receiving an "A" rating) and by relaxed criteria (receiving at least "B" ratings from both epidemiologists). PPVs were calculated by strict and relaxed criteria separately.

Results

Cluster Descriptions

The dataset contained a total of 6,382 positive cultures of any organism (from 3,346 different patients) from the units affected by the outbreak of oxacillin-resistant *S. aureus*. Of those, 728 (from 323 patients) were *S. aureus*. Of the 323 unique isolates of *S. aureus* in the affected units, 14 (4.3%) were oxacillin resistant, whereas for the hospital as a whole 84 (4.2%) of 1,983 *S. aureus* isolates were oxacillin resistant.

The dataset contained a total of 9,012 positive cultures of any organism (from 4,315 patients) from the units affected by the outbreak of vancomycin-resistant enterococcus. In the affected units, 21 (14.1%) of 149 enterococcal isolates were vancomycin resistant, whereas for the entire hospital 41 (5.3%) of 768 enterococcal isolates were vancomycin resistant.

For all implicated units, the 15 most common bacterial species represented 4,948 unique isolates, an average of 18 per unit per month. Overall 165 different organisms were isolated, 74 of them representing only three or fewer isolates over the 69 months included in the dataset.

CUSUMs

Several CUSUM charts proved capable of detecting both outbreaks by the second isolate. Figure 3 displays a representative CUSUM chart, which detected the VRE outbreak early in its course. Maximal performance robustness was obtained when 0.1 ≤ α ≤2 and 0.2 ≤ β ≤0.25, with p_0 = 0.05. Values of β <0.2 were associated with poor performance.

Monte Carlo simulations, run with p_1 = 0.2 over the sets of design parameters that performed most robustly, yielded an out-of-control TUD ranging from 8 to 45 isolates (average 20.4), and an in-control TUD, ranging from 55 to 2,390 isolates (average 427). Both the out-of-control TUD and in-control TUD decreased with higher values of α; for α = 0.2 or 0.25, the in-control TUD ranged from 55 to 88; whereas at α = 0.1, it ranged from 184 to 306 isolates.

The mean PPV of CUSUM techniques ranged from 96.5% (relaxed criteria) to 82.6% (strict criteria). Lower values for α were associated with higher PPV. On average, the sensitive control charts generated 9.5 novel alerts over the 69 months of the study period, or 1.6 events per year for all involved units and organisms (enterococcus, *S. aureus*).

Moving Averages

For MA control charts, only those which used quantitative MICs (vancomycin: 2–16 mg/mL; oxacillin: 0.25–4 mg/mL) were capable of detecting both outbreaks; no binary (susceptible = 0; nonsusceptible = 1) MA charts detected both outbreaks. Sensitive window sizes (w, the number of isolates considered in calculating the MA) varied from 5 to 30 isolates. Parameter sets with larger window sizes failed to detect both outbreaks.

Monte Carlo simulations for the design parameters that detected both outbreaks, assuming a change in MICs of one standard deviation, yielded an out-of-control TUD ranging from 4 to 10,796 isolates (mean 1,568; median 14), and an in-control

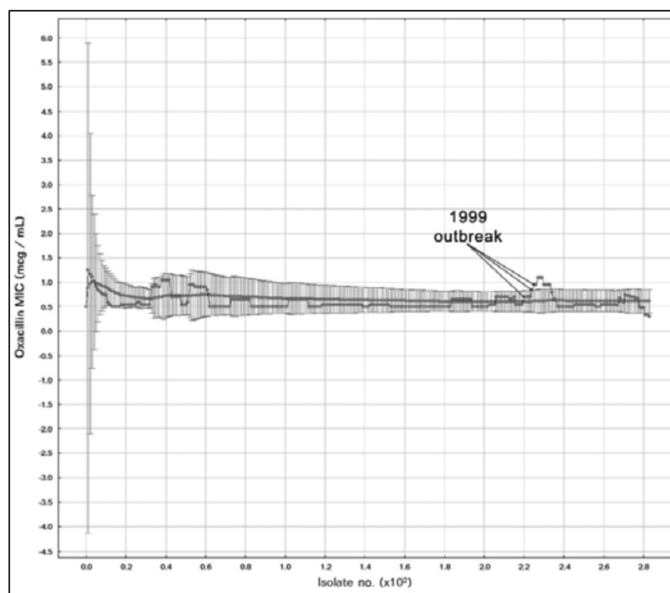

Figure 3. Moving average test iteration detecting an outbreak of methicillin-resistant *Staphylococcus aureus*. Test parameters were w = 10, k = 4, and included all *S. aureus* from all body sites from the affected wards, excluding strains found during outbreak investigations. MIC, minimum inhibitory concentration

TUD ranging from 11 to 25,488 (mean 4,006; median 180). For $k < 4$, the mean out-of-control TUD was 14, while the mean in-control TUD was 350 isolates.

Figure 4 displays a representative MA test combination that detected the MRSA outbreak by the second isolate. The mean PPV ranged from 88.5% (relaxed criteria) to 46.1% (strict criteria). On average, sensitive MA charts generated 10.9 novel alerts over the entire study period, or 1.9 per year for all units and organisms studied.

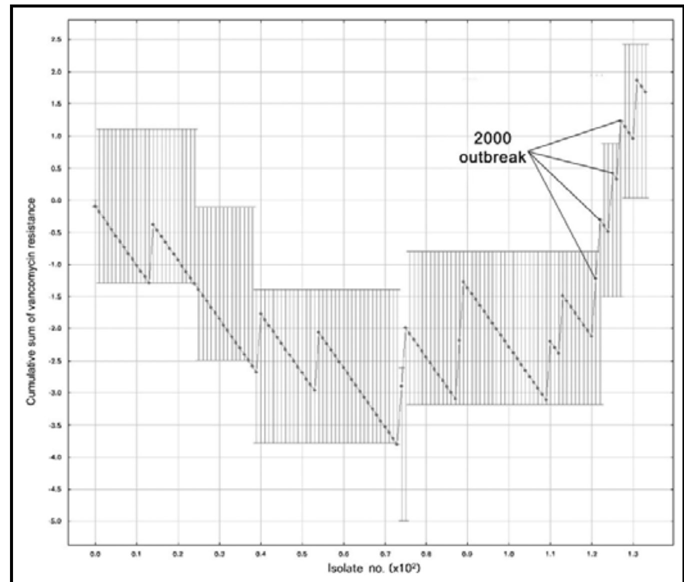

Figure 4. Cumulative sum test iteration detecting an outbreak of vancomycin-resistant enterococcus. Test parameters were p_0 = 0.05, p_1 = 0.15, a = 0.15, b = 0.2, and included enterococcal isolates from all body sites from the affected wards, excluding strains found during outbreak investigation.

Discussion

We illustrated the performance of a system designed for real-time monitoring of clinical microbiology data from the hospital laboratory information system. Two techniques borrowed from other domains were capable of detecting two carefully characterized outbreaks in simulated real time. The binary CUSUM proved more robust than MAs.

Many metrics for outbreak detection are based on month of outbreak (11,14,15,17,18,34), whereas in nosocomial outbreaks greater attention to individual cases is probably warranted given the smaller numbers of patients involved, the possibility of early definitive intervention, and the comorbidities of infected patients. The techniques used in this study proved capable of detecting an outbreak before the end of a monthly surveillance period.

The reproducibility of these findings is of key importance. We used an a priori reasonable set of possible design parameter values, then combined empirical evaluation of their performance with theoretical evaluation via Monte Carlo simulations.

We used only two outbreaks for evaluation, given the difficulty of generating and validating such datasets. A study that investigates larger numbers of similar outbreaks would improve generalizability. The theoretical simulations tend to support the generalizability of the test statistics used, as the empirically robust design parameters were associated with low out-of-control and high in-control TUD values.

The techniques appear most useful when the baseline incidence is relatively low, and it is unclear whether these methods would be applicable in settings where antibiotic-resistant bacteria are more common, as the study hospital had relatively low rates of MRSA and VRE.

The surveillance methods evaluated here are primarily useful for detecting outbreaks caused by resistant organisms. In their current implementation, they would not be useful for settings where outbreaks are caused by organisms whose antibiotic susceptibilities are indistinguishable from those of endemic flora, as in the cluster of *Pseudomonas* excluded from the present study. Additional research would be required to make these methods applicable in those settings.

From a practical perspective, the CUSUM charts detected the outbreaks by the second isolate, a finding corroborated by results of the Monte Carlo simulations. An increased incidence from .05 to .20 would be detected on average within 1.5 actual outbreak isolates for an out-of-control TUD of 10 (best-performing CUSUM), or at the third outbreak isolate for an out-of-control TUD of 20 (mean CUSUM performance). These results, supported empirically and theoretically, are consistent with the goals of nosocomial outbreak detection.

In terms of resources potentially wasted on false-positive results, the CUSUM charts that detected both outbreaks were remarkably accurate, with an average PPV of >80%, even by strict criteria, whereas the MIC MA parameter sets had lower PPVs. According to our calculated PPV for CUSUM, only 1 in 20 alerts would be deemed retrospectively as unworthy of any further evaluation, while 1 in 5 would not be deemed worthy of actual investigation. Assuming an annual rate of 1 alert per organism and unit, 4 units under surveillance, and 15 organisms under surveillance, 60 alerts would be generated annually, of which 12 would not be deemed worthy of attention, approximately one false alarm per month. Slightly more than twice as many would be considered spurious in retrospect on the basis of the MA results.

Using the in-control TUD values to estimate the frequency of spurious results yields a better estimate. With 18 isolates of the 15 most commonly isolated bacteria per unit per month, 4 units under surveillance, we would anticipate 72 isolates per month. The mean in-control TUD value for CUSUM charts is 427, suggesting a false-positive alert once every 5 months, though false-positive alerts are associated with a higher out-of-control TUD. Taking the chart with the lowest out-of-control TUD, the in-control TUD is 55, suggesting a false-positive result slightly more than once per month, similar to our observed rate.

Strengths of this study include the availability of genotyping data for outbreak characterization and the availability of quantitative MICs, the use of practical outcome measures, and

combination of empirical and theoretical methods for evaluating test statistics.

An additional problem in validating detection techniques is the lack of a gold standard for determining the relevance of a computer-detected cluster. We chose a practical approach, given the ultimate clinical application of such a system. We may have overestimated the positive predictive value, although we evaluated by both strict and relaxed criteria. At the time of evaluation, reviewers were unaware of events that followed, decreasing the probability of outcome-based bias. A prospective trial of these techniques, with collection of genotyping information, should help to resolve this problem.

Areas for additional research include methods for analyzing duplicate isolates from a single patient, more sophisticated techniques for modeling patient location, accounting robustly for changes in sampling intensity, methods for using quantitative CUSUMs, and the potential need for corrections for interdependence.

CUSUM and MA analyses of antimicrobial resistance proved capable of detecting two important nosocomial outbreaks early in their course in simulated real time. Both methods had relatively high positive predictive values; CUSUM performed better than MA. These analytical techniques may be of value in automated detection of nosocomial outbreaks and should be evaluated in real-time clinical practice.

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Dr. Brown is a resident in internal medicine at Massachusetts General Hospital. His research interests include nosocomial infections, antibiotic resistance, quality of medical care, outbreak detection, and infectious disease control in areas with limited resources.

Appendix: Calculating Test Statistics

Binary cumulative sum charts, based on the theory of sequential probability ratio tests, monitor a cumulative term that is incremented or decremented by certain amounts for each positive or negative result, respectively, in order to sequentially test between user-specified acceptable and unacceptable rates (35,36) (Equation 1). In our application, the CUSUM statistic S_i is reduced at the time of each isolate by an amount D, a calculated value that depends on the shift we wish to detect, and then increased by 1 for those isolates that are antibiotic resistant. The plotted statistic for the ith isolate, S_i, and the control limit factors h_0 and h_1 are calculated as

$$S_i = \begin{Bmatrix} S_{i-1} - D, & \text{if } X_i = 0 \\ S_{i-1} + 1 - D, & \text{if } X_i = 1 \end{Bmatrix} = S_{i-1} + X_i - D \text{, (1)}$$

$$h_0 = \frac{\ln\left(\frac{1-\alpha}{\beta}\right)}{\ln\left(\frac{p_1}{p_0} \cdot \frac{1-p_0}{1-p_1}\right)} \text{, and (2)}$$

$$h_1 = \frac{\ln\left(\frac{1-\beta}{\alpha}\right)}{\ln\left(\frac{p_1}{p_0} \cdot \frac{1-p_0}{1-p_1}\right)} \text{, (3)}$$

where $X_i = 1$ if the ith isolate is resistant and 0 if it is not, the decrement D is computed as

$$D = \frac{\ln\left(\frac{1-p_0}{1-p_1}\right)}{\ln\left(\frac{1-p_0}{p_0} \cdot \frac{p_1}{1-p_1}\right)} \text{,4}$$

α is the desired type I error rate, β is the desired type II error rate, p_0 is the acceptable occurrence rate, p_1 is the unacceptable occurrence rate that is desired to be detected, and $S_0 = 0$ as a starting value.

The cumulative sum then is compared to nonconstant control limits that periodically are recalculated by subtracting h_0 from and adding h_1 to any S_i value that falls outside either limit, resulting in new limits until the next such violation and starting with lower control limit (LCL) = $S_0 - h_0 = h_0$ and upper control limit (UCL) = $S_0 + h_1 = h_1$. Values above the UCL indicate an outbreak, i.e., rejection of the hypothesis of p_0 in favor of the hypothesis of p_1, although contrary to traditional control charts values beneath the LCL here do not indicate a rate decrease but rather acceptance of p_0 over p_1.

For the moving average (MA) charts, the moving average for the ith isolate with a "window" of size w (varied in different test conditions), $Y_{w,i}$, is calculated as

$$Y_{w,i} = \begin{Bmatrix} \frac{X_i + X_{i-1} + \ldots + X_{i-w+1}}{w} = \frac{\sum_{j=i-w+1}^{i} X_j}{w}, & \text{for } i \geq w \\ \frac{X_i + X_{i-1} + \ldots + X_1}{i} = \frac{\sum_{j=1}^{i} X_j}{i}, & \text{for } i < w \end{Bmatrix} \text{ (4)}$$

This result then is compared to estimated upper (UCL) and lower k-sigma control limits for the ith isolate, LCL_i and UCL_i, with the standard deviation of the ith moving average, $Y_{w,i}$, estimated by using the conventional moving range (MR) control chart method for individual data that occur over time,

$$\overline{MR}_i = \frac{\sum_{j=2}^{i} \left| X_j - X_{j-i} \right|}{i-1} \text{, (5)}$$

$$\hat{\sigma}_{Y,w,i} = \frac{\hat{\sigma}_{X,i}}{\sqrt{\min(i,w)}} = \frac{\overline{MR}_i / 1.128}{\sqrt{\min(i,w)}} \text{, (6)}$$

$$U\hat{C}L_i = \hat{\mu}_i + k\hat{\sigma}_{w,i} = \overline{X}_i + k\frac{\overline{MR}_i / 1.128}{\sqrt{\min(i,w)}} \text{, and (7)}$$

$$L\hat{C}L_i = \hat{\mu}_i - k\hat{\sigma}_{w,i} = \overline{X}_i - k\frac{\overline{MR}_i / 1.128}{\sqrt{\min(i,w)}} \quad (8)$$

all for $i \geq 2$, where i is the current total number of data points, X_i is the ith data value, w is the size of the moving average, and $\overline{X}_i$ is the average of all data up to and including the ith data value. An MA value that exceeds its corresponding UCL will trigger an outbreak alert.

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Using Automated Health Plan Data to Assess Infection Risk from Coronary Artery Bypass Surgery

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We determined if infection indicators were sufficiently consistent across health plans to allow comparison of hospitals' risks of infection after coronary artery bypass surgery. Three managed care organizations accounted for 90% of managed care in eastern Massachusetts, from October 1996 through March 1999. We searched their automated inpatient and outpatient claims and outpatient pharmacy dispensing files for indicator codes suggestive of postoperative surgical site infection. We reviewed full text medical records of patients with indicator codes to confirm infection status. We compared the hospital-specific proportions of cases with an indicator code, adjusting for health plan, age, sex, and chronic disease score. A total of 536 (27%) of 1,953 patients had infection indicators. Infection was confirmed in 79 (53%) of 149 reviewed records with adequate documentation. The proportion of patients with an indicator of infection varied significantly (p<0.001) between hospitals (19% to 36%) and health plans (22% to 33%). The difference between hospitals persisted after adjustment for health plan and patients' age and sex. Similar relationships were observed when postoperative antibiotic information was ignored. Automated claims and pharmacy data from different health plans can be used together to allow inexpensive, routine monitoring of indicators of postoperative infection, with the goal of identifying institutions that can be further evaluated to determine if risks for infection can be reduced.

Because postoperative surgical site infections are common complications of medical care, reducing their occurrence is a component of current efforts to improve patient safety. To guide these efforts and to measure their success, hospitals maintain resource-intensive programs to identify these infections (1–4). However, hospital-based programs detect only a minority of these infections. A principal contributor to the poor detection rates of hospital-based systems is the fact that a large majority of infections manifest after the patient leaves the hospital (5–13). Additionally, the substantial resources required to conduct prospective case detection requires some hospitals to monitor specific types of procedures only periodically, which means that hospitals may fail to detect problems that occur while they focus on other procedure types. Finally, variability in application of surveillance criteria has made comparing postoperative infection rates between hospitals difficult (4,14–16).

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We have described the use of diagnoses and procedures listed on automated billing data and of antibiotic prescriptions identified through automated pharmacy dispensing data to identify patients who are likely to have experienced postoperative surgical site infection both before or after discharge from the hospital (5,17,18). Overall, insurers' billing and pharmacy data identified substantially more patients with infection than did hospital-based surveillance in these studies.

These findings suggest that health plans' and insurers' routinely collected billing data might be used to supplement hospital-based programs. More importantly, this information might be used to compare different hospitals' results. However, the comparability of data from different health plans for this purpose has not been assessed. Nor has any attempt been made to combine information from different health plans; information from several health plans is often necessary because the number of procedures for persons in one health plan is usually too small to allow acceptably precise estimates of risk. Health plans differ in type, detail, quality, and completeness of the information they collect and maintain. For example, health plans vary widely in their prescription drug coverage and, therefore, in the amount of information they possess about their members' antibiotic exposure.

We assessed the usefulness of using data from several large health plans to detect patients who may have had an infection after coronary artery bypass grafting; we wanted to know if

this information could be used to compare the experience of different hospitals. Additionally, we assessed the contribution of pharmacy data to these comparisons to determine the potential for using data from persons without pharmacy benefits.

Methods

Study Population

We studied patients who had coronary artery bypass graft (CABG) surgery from January 1996 through March 1999 and who received health-care coverage from Harvard Pilgrim Health Care, Tufts Health Plan, or Blue Cross Blue Shield of Massachusetts. Harvard Pilgrim and Tufts Health Plan (including Secure Horizons, Tufts Health Plan for Seniors, a Medicare + Choice managed care plan) are HMOs. Blue Cross included HMO and indemnity plans. Together, these organizations accounted for approximately 90% of managed care in eastern Massachusetts during that time. We focused principally on procedures performed in four hospitals, which were members of the Eastern Massachusetts Prevention Epicenter, and which performed CABG. These institutions performed a majority of the CABG procedures in the region. CABG procedures at Harvard Pilgrim through June 1997 were described in a separate report, and so are omitted here. Patients were excluded who had a second CABG procedure within 30 days of a previous procedure.

Although we wanted to restrict the population to persons with prescription drug coverage, some of the HMOs could not provide coverage status at the time of surgery. Therefore, we excluded from our main analysis any persons for whom the HMO had no claims for any prescription drugs for 180 days before the date of surgery. Among patients for whom pharmacy benefit status was provided, pharmacy dispensing (at least one prescription) 180 days before surgery correlated with having benefits; 97% of those with some dispensing had a drug benefit compared to 19% with no drug benefit.

Data Sources

Automated Data

At the time of the study, Tufts Health Plan maintained two administrative claims systems: one for its commercial plan and another for a Medicare plan. Harvard Pilgrim maintained three legacy systems: one derived from a staff model HMO, one from its network and group division, and one from an IPA system. In total, six claims systems were used. HMO staff searched these six claims systems for hospital claims with ICD9 procedure codes 36.10–36.16, 36.19, or CPT codes 33510–14, 33516–19, 33521–23, 33533–36 in physician billing records. For convenience, we refer to each of these claims systems as a separate plan (i.e., plan 1 through plan 6). These separate divisions had generally similar benefits (e.g., covering ambulatory services and prescription drugs except for a copayment required of the patient). However, one of the plans changed its coverage during the study period, including a capped pharmacy benefit for every 3-month period and a period of no pharmacy coverage.

For all patients with a procedure code of interest, each health plan provided the patient's age and sex. The health plans also provided inpatient and outpatient claims from the index hospitalization through 30 days after the date of the surgical procedure. For each claim, they provided an encrypted patient identifier, a date of service, a service location (inpatient, skilled nursing facility, emergency room, or outpatient), and all diagnosis and procedure codes. For each prescription, they provided the same patient identifier, the date of dispensing, and a drug identifier (all provided National Drug Code and generic names). Some HMOs provided data for all CABG procedures. Others provided data only for patients whose CABG procedure was performed at an Epicenter hospital. However, in those cases they provided follow-up data from any hospital. We tested the completeness of the claims files in several ways (e.g., we assessed the number of days per patient on which ambulatory services were provided and the number of diagnoses listed on such days). We excluded health plans from our main analyses if their data appeared to be incomplete. The specific reasons for exclusion are described.

For each patient, we identified all claims with any codes suggestive of surgical site infection (Appendix 1). We refer to the codes in Appendix 1 as indicators of infection. These codes included inpatient diagnoses of infection, ambulatory diagnoses of infection, procedure codes suggestive of infection, and antibiotics dispensed in the ambulatory setting. This list is a more general set of codes previously identified as being important (17,18); we added closely related codes that had not appeared in our earlier datasets. We also assigned an estimated probability that infection had occurred by using a previously described algorithm developed in a dataset that included a broad range of surgical procedures, including coronary artery bypass (17,18, Appendix 2). Because the algorithm assigns a higher baseline probability of infection to patients undergoing cardiac surgery than those undergoing other procedures, 536 (97%) of 550 patients with any of the indicators in Appendix 1 had an estimated probability of infection exceeding 9.5%. Although we sought to confirm the infection status of the 536 with probabilities exceeding 9.5%, we refer to them as patients with any indicator, since this has a functionally equivalent meaning for patients undergoing CABG. Identifying patients with any indicator is much simpler than identifying patients who exceed a threshold predicted probability of infection.

Medical Record Information

We attempted to obtain the medical records of patients who had claims with an indicator of infection. Because most patients received care from a variety of providers in different facilities, we requested the record of the first provider or facility that submitted a claim with an indicator of infection. In many cases, identifying an institution to request records from was not possible. In these cases, we requested the record from

the patient's primary care physician. For logistical reasons, reviewing records of patients belonging to one of the health plans was not possible.

Patients' records with an indicator code were reviewed by trained abstractors for evidence of postoperative surgical site infections, by using the criteria from the Centers for Disease Control and Prevention's National Nosocomial Infection Surveillance System (CDC NNIS) (19). The reviewers also noted if the information in the medical record was adequate to judge the presence of infection. Typical reasons that records were judged inadequate included inappropriate date range of the records provided or lack of indication that the patient had received postoperative care from the provider. An infectious disease specialist reviewed records with evidence of abnormal wound healing and classified the outcome as confirmed infection, abnormal wound that met some criteria for infection, or no evidence of infection.

Chronic Disease Score

We computed a chronic disease score (the Clark TC score) (20,21) for each patient, for use as a comorbidity adjuster. Components of the chronic disease score include the patient's age, sex, and prescription medications during the previous 6 months. Points are assigned for 29 diseases or disease categories (i.e., diabetes, if the patient has any dispensing of a drug typically used to treat the disease). The chronic disease score has been shown to predict hospitalization and also to correlate with the risk for postoperative surgical site infection in a general surgery population (22,23).

Data Analysis

Simple comparisons of categorical data were performed by chi-square testing. Continuous variables were often not normally distributed and were compared by nonparametric tests, either the Wilcoxon if two groups were being compared or the Kruskal-Wallis test for more than two. The strength of correlation between continuous variables was assessed with the Spearman correlation coefficient. Logistic regression was used to investigate a central question in the current investigation (i.e., if enough consistency existed between plans to allow comparisons between hospitals). This question was assessed by using a hospital-by-plan interaction term in the model. The model also assessed and controlled for the relative contribution of health plan, hospital, age, sex, and chronic disease score to the probability of individual patients having a claim suggestive of infection.

Results

These health plans provided data for 3,014 CABG procedures performed from January 1996 through March 1999. A total of 858 patients had no claims for prescription drugs for 180 days before surgery, 46 had claims in two different claims systems, 39 had an uncertain procedure type, and 7 had a second CABG within 30 days. In addition, one of the plans was unable to provide claims for postoperative ambulatory care for 99% of its patients; claims from this plan were determined to be unusable because they were incomplete. All 252 persons represented by this claim system were excluded. We excluded the 1,061 patients with at least one of these criteria from our main analyses (some patients met more than one exclusion criterion). The total number of included procedures was 1,953, representing 65% of all procedures. The median age was 61 years, 78% were men, a median of 15 prescriptions were filled 6 months before surgery, and the median chronic disease score was 2,283 (Table 1). Postoperatively, a median of five prescriptions were filled in 30 days after surgery. Substantial differences existed between the health plans in members' age, sex, chronic disease score, and number of prescriptions before surgery. In the 30 days after surgery, substantial differences existed in the number of prescriptions (all drugs) and number of days that patients received ambulatory care.

Overall, based on claims data alone, at least one indicator code for surgical site infection was found in 536 (27%) of 1,953 patients, with a range of 22% to 33% in the different health plans (Table 2). In patients with at least one such indicator code, the estimated probability that infection had occurred, based on our algorithm, was tightly clustered in two ranges: one was approximately 10% and the other approximately 70%. The distribution of estimated probabilities for all patients together and separately by health plan is shown in Figure 1. The overall pattern was similar across the health plans, although the distribution of probabilities was significantly different among them ($p<0.01$, Kruskal-Wallis). The specific types of indicators that contributed to patients being classified as high risk and the locations in which they occurred are shown in Table 1. Forty-nine (3%) patients had an infection indicator code during initial hospitalizations. In the 30 days after surgery, 77 (4%) persons had an indicator during a second hospitalization; 48 (62%) of second hospitalization occurred at the same institution in which surgery had been performed. Forty-three (2%) patients had an infection indicator during an emergency room visit, 280 (14%) had an indicator during an ambulatory-care visit, and 291 (15%) were dispensed an antistaphylococcal antibiotic (Table 1). Statistically significant differences occurred across health plans in the percentages of patients who had indicator diagnoses during initial hospitalization ($p=0.05$) and rehospitalization ($p<0.01$), who had claims for wound cultures ($p<0.01$) and wound care ($p=0.052$), and who received antistaphylococcal antibiotics ($p=0.05$) but not in diagnoses in emergency rooms or other ambulatory settings.

We requested full text medical records that had infection indicator codes for 368 patients who were members of plans that participated in the record review component of this study. We obtained 275 (75%) of these (Table 3). The health plan with automated ambulatory medical records retrieved nearly all requested records; from the others, the proportions ranged from 66% to 79%. From records obtained, 149 (54%) contained sufficient information to allow assessment of the presence or absence of postoperative surgical site infection.

Table 1. Characteristics of coronary artery bypass patients of five health-care plans in Massachusetts, January 1996–March 1999

	Plan 1	Plan 2	Plan 3	Plan 4	Plan 5	All	p value[a]
Procedures	161	584	635	363	210	1,953	
Age (range)[a]	67 (61–73)	59 (53–64)	60 (54–65)	59 (53–63)	72 (68–75)	61 (55–67)	<0.0001
Sex, % male	77	78	83	80	60	78	<0.0001
Prescriptions (range) during the 180 days before surgery[b]	14 (9-22)	18 (10-28)	18 (12-27)	9 (6-12)	14 (7-21)	15 (9-24)	0.0001
Chronic disease score (range)[b]	2,474 (1,774–3,704)	2,211 (1,403– 3,456)	2,378 (1,506–3,662)	2,156 (1,372–3,350)	2,369 (1,432–3,376)	2,283 (1,446–3,511)	0.01
Prescriptions (range) during the 30 days after surgery [b]	5 (3–7)	6 (4–8)	4 (3–6)	5 (3–7)	3 (0–5)	5 (3–7)	<0.0001
Days (range) with ambulatory care claims [b]	1 (1–2)	10 (7–13)	6 (3–8)	11 (8–16)	14 (10–18)	8 (5–13)	0.0001
Diagnoses on days with ambulatory claims (average)	2.6	2.6	2.2	1.9	1.5	2.2	
Patients with specified indicator of infection % (no. of patients):							
Diagnosis during index hospitalization	6 (9)	3 (17)	2 (14)	2 (7)	1 (2)	3 (49)	0.05
Diagnosis in ambulatory setting (excludes emergency room)	12 (19)	15 (89)	17 (105)	11 (39)	13 (28)	14 (280)	NS
Diagnosis in emergency rooms	2 (3)	3 (19)	2 (12)	2 (6)	1 (3)	2 (43)	NS
Diagnosis during second hospitalization, includes extended care facilities	9 (14)	7 (41)	2 (15)	2 (6)	0 (1)	4 (77)	<0.01
Antistaphylococcal antibiotic in ambulatory setting	16 (26)	18 (107)	13 (81)	14 (52)	12 (25)	15 (291)	0.05
Wound culture performed	2 (3)	2 (12)	5 (33)	6 (22)	1 (2)	4 (72)	<0.01
Wound care	0 (0)	1 (4)	2 (15)	1 (5)	1 (2)	1 (26)	0.052

[a]Kruskal-Wallis or chi-square tests of the null hypothesis of no difference between plans.
[b]Median (interquartile range).

Common reasons for classifying the documentation inadequate were lack of any evidence that the provider or facility had cared for the patient during the 30 days after surgery or submission of records from a time that excluded this interval. Records were considered to be adequate if the status of the incisions during a postoperative visit was not mentioned. From charts with adequate documentation, 79 (53%) patients were confirmed to have infection; 70 of these infections were superficial. Another 19 (13%) patients partially satisfied CDC NNIS criteria for surgical site infection. The confirmation rate was similar for those with estimated probabilities of infection of >9%–20% (48%, 35/73) and those with estimated probabilities >50% (59%, 43/73). No substantial difference existed between either health plans or hospitals in the proportions with confirmed infection; these proportions exceeded 50% for every health plan and hospital except one, for which the confirmation rate was 45% (data not shown).

Of the four studied hospitals, the proportions of patients with an indicator of infection varied from 19% to 36% (Table 2). The rank ordering of the hospitals was consistent across the different health plans, with hospital D having the highest proportion in four plans and hospital B having the lowest in three of them. Hospital D's excess, compared to hospitals A and B, was considerably greater during the year beginning April 1997 than during the year beginning April 1998 (Figure 2). Hospital B had either the lowest percentage or was close to the lowest during the 2 years. After these results were known, hospital D indicated that it had identified an increase in its sternal surgical site infection rate from July through December 1997 through hospital-based surveillance and had intervened to address this

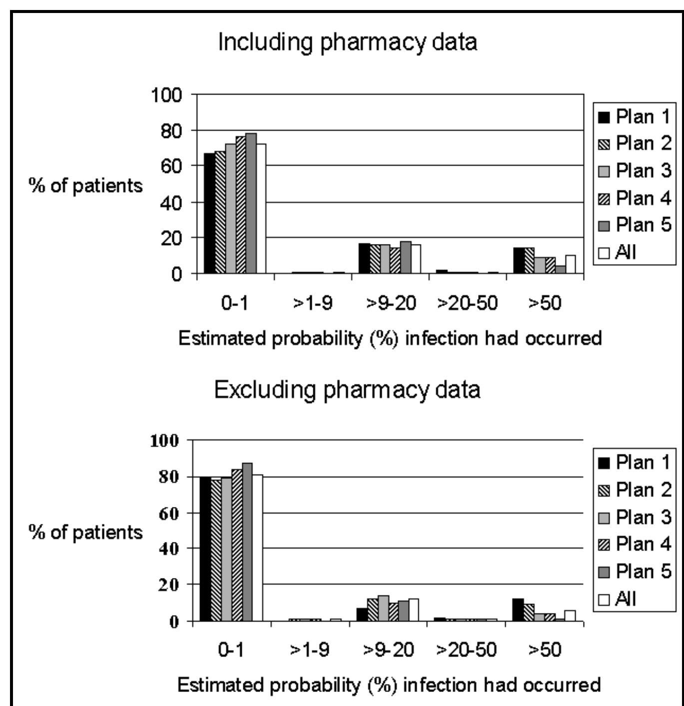

Figure 1. Distribution of individual patients' estimated probability of infection.

Table 2. Patients with indicators of infection by hospital and health maintenance organization claims system

	Plan 1	Plan 2	Plan 3	Plan 4	Plan 5	Total
Hospital	(% Patients with indicator/all patents)					
A	33 (36/108)	33 (45/136)	27 (48/175)	23 (12/53)	16 (7/45)	29 (148/517)
B	—	15 (7/41)	21 (54/256)	16 (21/131)	25 (8/32)	19 (89/460)
C	30 (3/10)	56 (9/16)	26 (16/62)	25 (12/48)	11 (3/28)	26 (53/164)
D	100 (1/1)	41 (41/100)	55 (50/141)	34 (29/86)	34 (11/32)	36 (132/360)
Other	31 (13/42)	28 (81/291)	0 (0/1)	27 (12/45)	25 (18/73)	27 (124/452)
All	33 (53/161)	31 (182/584)	26 (168/635)	23 (86/363)	22 (47/210)	27 (536/1953)

increase (pers. comm., hospital D's epidemiologist). The increase noted by hospital D overlapped with the two periods during which claims-based surveillance showed the hospital's rate to be high.

The hospitals were different from one another in the proportion of patients with an indicator of infection (p<0.0001), after controlling for health plan, patient age, and patient sex. The adjusted relative odds of a patient's having an indicator for infection for hospital D compared to hospital B was 2.3, with intermediate values for the others (Table 4). Patient age, sex, and health plan were also significant predictors of an indicator of infection. However, the interaction between health plan and hospital was not significant, indicating that the risk of infection at each hospital was not affected by membership in any particular plan. The adjusted relative risks for the hospitals were nearly identical in models that substituted the chronic disease score, which incorporates preoperative prescription drug information along with age and sex, in place of age and sex as separate risk factors. Nearly identical results were obtained when the health plans with highest and lowest values were excluded from these analyses, either singly or together. The results were also nearly the same when we included the 969 patients (totaling 2,922 of the original 3,014 cases) who had been excluded because they had no pharmacy dispensing activity during the 6 months before surgery or because they belonged to the health plan that provided no claims for postoperative ambulatory care.

We also assessed the effect of ignoring postoperative antibiotic information. In this situation, 363 (18%) of 1,953 persons had an indicator suggestive of infection, compared to 27% when antibiotics were included, using the same model and setting the contribution of absolute zero. The distribution of estimated probability that infection had occurred still had two peaks, clustered as before at 10% and 70% (Figure 1). These estimated probabilities including and excluding postoperative antibiotic information were highly correlated. For all patients together, the Spearman correlation coefficient was 0.81 (p=0.0001); the health plan specific correlation coefficients ranged from 0.71 to 0.83. When postoperative antibiotic dispensing in the ambulatory setting was ignored, qualitatively similar results regarding the relative odds associated with specific hospitals were also obtained, although the effect of the hospital was less strong (p=0.03, Table 4).

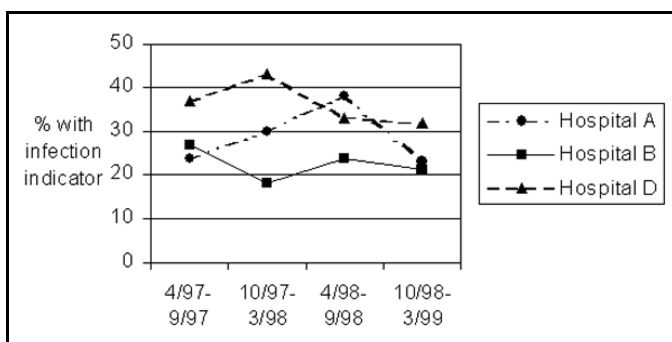

Figure 2. Proportion of patients with an indicator of infection, by hospital and 6-month period.

Discussion

These results agree with earlier findings indicating the value of using automated claims data to identify persons who are likely to have experienced a postoperative surgical site infection. In this setting, infection was confirmed in approximately 58% of patients with an indicator in claims data. These findings were also consistent with earlier findings that most infections are detected in ambulatory settings or in hospitals other than the one in which surgery was performed. This result is notable in this case because complications of CABG are probably more likely to be cared for in the institution where surgery is performed, compared to complications of other types of procedures.

A principal reason to use automated data in this way would be to screen institutions periodically to identify those with higher than expected proportions of patients with an indicator of infection. However, a high proportion of patients with indicators does not necessarily imply that a hospital's infection rate is high, since the overall confirmation rate may vary across hospitals and over time. Rather, the finding of a high proportion with infection indicator codes would allow directed inquiry about whether these institutions' actual infection rates exceeded either their own usual level or the rates for similar institutions. In our data, the claims data suggested that hospital D's rate was high, which was confirmed by the hospital. These data might also be used to identify institutions with consistently low proportions of patients with indicator codes; these institutions may be able to assist others in identifying and implementing best practices. In our case, hospital B may be

Table 3. Infections noted in full text medical record review[a]

	Plan 1	Plan 2	Plan 4	Plan 5	All
Records sought	53	182	86	47	368
Records obtained (%)	51 (96)	125 (69)	68 (79)	31 (66)	275 (75)
Adequate documentation (% of records received)	45 (88)	62 (50)	29 (43)	13 (42)	149 (54)
Adequate documentation among records sought	45 (85)	62 (34)	29 (34)	13 (28)	149 (40)
Surgical site status (% of those with adequate documentation)					
Confirmed surgical site infection	23 (51)	31 (50)	15 (52)	10 (77)	79 (53)
Problem wound healing, not meeting criteria for infection.	7 (16)	7 (11)	3 (10)	2 (15)	19 (13)
No evidence of infection	15 (33)	24 (31)	11 (38)	1 (8)	51 (34)

[a]For logistical reasons, records were not sought from plan 3.

such an institution, since its proportion of patients with an indicator code was usually the lowest of the group.

Since claims like the ones used here are created for nearly all patients, performing such screening for most hospitals would be possible. The type of work involved, manipulation of automated claims data and review of selected records, is similar to work already performed by many health maintenance organizations as part of their accreditation requirements. Such activities might be integrated with those of peer review organizations, which have experience in working with hospitals to assess care and to implement changes to improve it. The incremental work for health plans of performing this screening is relatively small, after the initial work of implementation.

The data we used were created mainly to support financial operations; therefore, the underlying data systems differ considerably within and between health plans. Health plans' data systems differ (e.g., the number of diagnoses and procedures per claim that they capture). Additionally, the reliability of data can vary in ways not appreciated by the health plans themselves. For example, we found that one health plan identified no claims for postoperative ambulatory care. Limiting assessment to patients and procedures for which the overall patterns of care appear to be appropriate is important.

Health plans themselves differ in a variety of ways that may influence the proportion of patients with indicators for infection, including the patient populations they serve. These populations may differ in their underlying risk for infection. In the health plans we studied, the different distributions of age, sex, and chronic disease scores illustrate this point. The different benefits packages of the health plans also cause differences in the proportion with indicators. For example, one of the health plans had limited prescription drug coverage. Therefore, comparisons between health plans' results must be made with care.

The differences between the health plans did not affect our inter-hospital comparisons, shown in Table 4. We interpret this result to mean that combining results from different health plans is possible, as long as the comparisons control for health plan. Combining results to obtain sufficient numbers of procedures and stable estimates of risk is desirable. Consistency of effect across insurers provides additional support for the comparisons.

Differences between hospitals' proportions of patients with infection indicator codes may reflect a difference in the actual risk of infection, but they may also result from systematic differences in the way they or their clinicians assign diagnosis and procedure codes or report them to payers. Because of these potential differences, outlier values observed in claims data should be confirmed by direct assessment of clinical outcomes. For this reason and others, a hospital's proportion of patients with indicator codes should not be equated with its infection rate.

Because our record review confirmed similar proportions of infections among patients with low and high estimated risks of infection, we recommend focusing on all patients with any of the indicators, rather than those with higher estimated probabilities of infection. Focusing on all patients with an indicator of infection eliminates some of the potential sources of unin-

Table 4. Adjusted hospital specific risks[a]

	Patients with at least one indicator code for infection	Hospital B vs. A	Hospital C vs. A	Hospital D vs. A	Other hospitals vs. A	p value
Including pharmacy data	536/1,953	0.68 (0.49–0.94)[b]	1.03 (0.68–1.55)	1.57 (1.16–2.13)	0.91 (0.67–1.24)	<0.0001
Excluding pharmacy data	363/1,953	0.84 (0.58–1.20)	0.92 (0.56–1.50)	1.62 (1.15–2.28)	1.05 (0.74–1.50)	0.03

[a]Adjusted for health plan, age, and sex. The interaction between health plan and hospital was not significant in any of these models. Similar results were obtained in models adjusting for chronic disease score (a composite of age, sex, and pharmacy information), instead of age and sex.
[b]Adjusted odds ratios and (95% confidence intervals).

formative variation between hospitals and health plans, since the estimation of risk for each patient uses combinations of specific codes. In addition, the larger number of events provides more stable estimates and therefore facilitates comparisons. The relatively small number of confirmed deep infections prevented assessment of differential ability of this method to identify different types of surgical site infections. We do not know whether the fact that 89% of confirmed infections were superficial reflects the actual epidemiology of surgical site infections or differential ascertainment of deep infections, which are likely to be diagnosed and treated in the inpatient setting. Our earlier work (18) suggested the approach we used had good sensitivity for detecting infections diagnosed among inpatients.

Although pharmacy claims are an indicator of infection, sometimes the only indicator, we obtained qualitatively similar results when we ignored this information. Therefore, we believe claims data can be assessed even when pharmacy claims are unavailable. However, controlling for the availability of this information will be important when making comparisons. Although automated ambulatory medical records are still not widely used, using their information when it is available is worthwhile, since this information is typically more complete. The same caveat will apply about controlling for automated medical records versus claims data as the data source.

We cannot directly extrapolate these results regarding coronary artery bypass to other types of procedures. For instance, ICD9 code 998.5 (postoperative infection) may be much less specific when assigned during hospitalizations for other types of surgical procedures. For example, the code may be assigned during a hospitalization for breast surgery to treat pre-existing infection. Because of this, the usefulness of these indicators for common procedure types should be determined. However, earlier work did indicate usefulness of indicators obtained from automated data for an unselected set of nonobstetric procedures (17) and separately for identification of postpartum infection (23).

We conclude that automated claims systems currently maintained by most health plans and insurers to reimburse institutions, providers, and pharmacies can serve as the basis for a screening system that would allow assessment of most hospitals' outcomes of coronary artery bypass procedures and possibly of other procedure types. Such screening would allow focused follow-up of specific institutions to determine if their infection rates actually are high and if specific practices can be changed to reduce the risk to patients. Such screening systems will be particularly useful if different health plans combine their results. The effort required to implement a system that includes a majority of hospitals that perform coronary artery surgery is not large in relation to existing quality improvement programs and would provide information that complements existing programs for identification and control of surgical site infections.

Acknowledgments

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Dr. Platt is professor of Ambulatory Care and Prevention at Harvard Medical School and Harvard Pilgrim of Health Care. He is also hospital epidemiologist at Brigham and Women's Hospital, Boston. His interests include surveillance and prevention of infectious disease and education and research on therapeutics.

Appendix 1.

Antibiotics, diagnosis, and procedure codes used to identify potential infections[a]

Antibiotics[b]

Cephalexin
Dicloxacillin
Clindamycin
Cephradine
Vancomycin

Diagnoses assigned in hospitals, emergency departments, or outpatient settings (ICD9 codes)

Code	Description
998.0	Postoperative Shock
998.3	Post-op Wound Disruption
998.5	Postoperative Infection
998.51	Infected Post-op Seroma
998.59	Post-op Infection Nec
998.83	Non-Healing Surg Wnd
780.6	Fever
891.0	Op Wnd Low Leg /S Comp
891.1	Open Wnd Knee/Leg-Comp
682.6	Cellulitis of Leg
682.9	Cellulitis Nos
998.9	Surgical Comp Nos
38.0	Streptococcal Septicemia
38.1	Staph Septicemia
38.10	Staph Septicemia Nos
38.11	Staph Aureus Septicemia
38.19	Staph Septicemia Nec
38.2	Pneumococcal Septicemia
38.3	Anaerobic Septicemia
38.4	Gram-Neg Septicemia Nec
38.40	Gram-Neg Septicemia Nos
38.41	*H. influenzae* Septicemia
38.42	*E. coli* Septicemia
38.43	Pseudomonas Septicemia
38.44	Serratia Septicemia
38.49	Oth Gram-Neg Septicemia
38.8	Septicemia Nec
38.9	Septicemia Nos
790.7	Bacteremia
611.0	Inflam Disease of Breast
682.0	Cellulitis of Face
682.1	Cellulitis of Neck
682.2	Cellulitis of Trunk
682.3	Cellulitis of Arm
682.4	Cellulitis of Hand
682.5	Cellulitis of Buttock
682.6	Cellulitis of Leg
682.7	Cellulitis of Foot

682.8	Cellulitis, Site Nec
682.9	Cellulitis Nos
686.0	Pyoderma
686.1	Pyogenic Granuloma
686.8	Local Skin Infection Nec
686.9	Local Skin Infection Nos
958.3	Posttraum Wnd Infect Nec
711.00	Pyogen Arthritis-Unspec
996.6	Infect/Inflam-Dev/Graft
996.60	Infect Due To Device Nos
996.61	Infect D/T Hrt Device
996.62	Infect D/T Vasc Device
996.63	Infect D/T Nerv Device
996.64	Infect D/T Urethral Cath
996.65	Infect D/T GU Device Nec
996.66	Infect D/T Joint Prosth
996.67	Infect D/T Orth Dev Nec
996.68	Infect D/T PD Cath
996.69	Infect Due To Device Nec
674.3	Oth Comp OB Surg Wound
879.0	Open Wound of Breast
879.1	Open Wound Breast-Comp
879.2	Opn Wnd Anterior Abdomen
879.3	Opn Wnd Ant Abdomen-Comp
879.4	Opn Wnd Lateral Abdomen
879.5	Opn Wnd Lat Abdomen-Comp
879.6	Open Wound of Trunk Nec
879.7	Open Wnd Trunk Nec-Comp
879.8	Open Wound Site Nos
879.9	Opn Wound Site Nos-Comp
875.0	Open Wound-Chest/S Comp
875.1	Open Wound Chest-Comp
Specimens obtained for culture (CPT codes)	
87040	Blood Culture for Bacteria
87072	Culture of Specimen by Kit
87075	Culture Specimen, Bacteria
87076	Bacteria Identification
87081	Bacteria Culture Screen
87082	Culture of Specimen by Kit
87083	Culture of Specimen by Kit
87084	Culture of Specimen by Kit
Wound care procedures (CPT codes)	
10180	Complex Drainage Wound
11000	Debride Infected Skin
11001	Debride Infect Skin Add
15852	Dressing Change, Not for Burn

[a]Some of these codes are applicable principally to surgical procedures other than CABG. They were included during the development of the algorithm shown in Appendix 2, which was developed to include CABG and other procedures [XXREF]. For consistency, these codes were retained in this evaluation of CABG procedures alone.

[b]These antibiotics were identified by an ambulatory pharmacy claim.

Appendix 2.

Algorithm used to assign probability that infection had occurred

$p = \exp(\alpha + {}_1x_1 + \ldots + {}_nx_n)/(1 + \exp(\alpha + {}_1x_1 + \ldots + {}_9x_9))$, where

$\alpha = -5.16$

$\beta_1 = +3.03$

x_1 = dispensing any of five selected antibiotics

$\beta_2 = +6.06$

x_2 = any selected diagnosis in hospital

$\beta_3 = +1.05$

x_3 = any selected diagnosis in emergency dept (if x2 = 0)

$\beta_4 = +2.98$

x_4 = any selected diagnosis in outpatient setting

$\beta_5 = +2.91$

x_5 = selected bacterial culture

$\beta_6 = +1.91$

x_6 = wound care

$\beta_7 = -1.79$

x_7 = interaction of x4 and x6

$\beta_8 = -2.70$

x_8 = interaction of x4 and x2

$\beta_9 = -2.21$

x_9 = interaction of x4 and x5

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RESEARCH

Cross-Sectional Study on Influenza Vaccination, Germany, 1999–2000

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To assess influenza vaccination coverage in Germany, we conducted a nationwide telephone survey in November 1999 in adults (≥18 yrs) using random-digit dialing. Overall, 23% of 1,190 survey participants reported having been vaccinated (adjusted 18%) with 16% (adjusted 15%) in former West Germany versus 35% (adjusted 32%) in former East Germany. Immunization rates for vaccination target groups were lower in West Germany (21%) than in East Germany (40%). Seven percent of health-care workers were immunized. Previous influenza vaccination, positive attitudes towards immunization, and having a family physician increased the rate of vaccination; fear of adverse effects lowered the rate. Family physicians performed 93% of the vaccinations, which suggests their key role in improving low vaccination coverage in Germany. The fact that >71% (850/1,190) of participants belonged to at least one of the vaccination target groups recommended by the German Standing Commission on Immunization emphasizes the need to focus the definition of target groups.

Ten years after the reunification of the former East and West Germany, the Federal Republic of Germany still shows the effects resulting from combining two different health-care systems after being apart for 50 years. Even though the health-care systems were merged soon after reunification, differences in health-care practices persist, especially in regard to preventive medicine and immunization, which had a much higher priority in the former East Germany. For example, a pilot study undertaken in Berlin, Stuttgart, and Chemnitz during the influenza season 1998–1999 showed much higher influenza vaccination rates in East Berlin and Chemnitz in the former East Germany (called former East in this paper) than in West Berlin and Stuttgart in the former West Germany (called former West in this paper) (1,2).

In general, population-based studies of influenza vaccination coverage for a country do not exist. A Canadian study found 13.8% influenza vaccination coverage in fall and winter 1990–1991 (3). Most studies on influenza vaccination coverage investigate specific groups such as the elderly (4–13), patients from general practices (14,15), or hospitalized patients (16).

This lack of nationwide, population-based studies, along with the findings of the pilot study showing markedly different vaccination rates in several German cities, prompted the nationwide, population-based survey reported here. The goals of our survey were to determine the influenza immunization rates in areas of the former East and West during the 1999–2000 influenza season, the proportion of the German population included in specific vaccination target groups recommended by the German Standing Commission on Immunization, the vaccination rates among these target groups, and factors that might influence immunization rates in areas of the former East and West.

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Methods

Background

Germany has a population of 82 million; 14 million live in areas of the former East and 68 million in areas of the former West (17). The German Standing Commission on Immunization has recommended that the following groups receive influenza vaccination: 1) persons >60 years old, 2) persons with chronic illness, 3) health-care professionals, and 4) persons who have extensive contact with the general public. The first three groups comprise an estimated 35% of the general population and 42% of the adult population of Germany (Arbeitsgruppe Seuchenschutz, Robert Koch-Institut, 7 September 1999). The size of the fourth group is unclear because of its widely applicable definition. For the target groups, the influenza vaccination period started in September 1999. Vaccinations were administered free of charge.

Survey

The target survey population included noninstitutionalized persons ≥18 years of age living in Germany. A standardized, pretested questionnaire was administered by telephone on November 8 and November 22, 1999. Sample households were chosen by random-digit dialing by using a computer-generated list of possible telephone numbers. Approximately half of the telephone numbers on the list had prefixes in the former East. However, the proportion of working phone numbers was

lower in the former East, so the actual number of former East residents who answered the phone and agreed to participate was <50% of all participants. The person who initially answered the telephone was eligible to be interviewed; to be eligible, persons also had to be ≥18 years of age, live in a private household, and have sufficient knowledge of German to be able to understand and answer the questions. If persons <18 years of age answered the phone, they were asked if an adult was present in the household, and an attempt was made to interview that person. After verbal, informed consent was obtained from the participant, we administered a questionnaire that gathered information about demographics, individual risk factors for contracting influenza, history of vaccination, general attitude towards immunization, perceived efficacy and adverse effects of the influenza vaccine, as well as other factors that might influence whether a person was likely to have been vaccinated.

Participants were counted as being vaccinated in the current influenza season if they reported having received an influenza vaccination after September 1, 1999. Persons were counted as being in a target group recommended by the German Standing Commission on Immunization if they reported one or more of the following: 1) age of ≥60 years, 2) chronic illness currently requiring regular medical supervision or treatment, 3) work in a health-care environment, in which at least half the working day involved interacting with patients, and 4) working at a job in which more than half the working day was spent with people who were not their colleagues. To provide added specificity, these latter two groups were narrower in scope than those defined by the Standing Commission on Immunization. In the following text, these two groups are summarized as "professional exposure." Immunization rates were adjusted for age, sex, and residence in areas of the former East and West according to the official 1998 population data (17).

Results

Study Population

Dialing 4,863 numbers yielded 2,057 actual connections. Of these, 25 were discarded because the person who answered the phone had insufficient knowledge of German and 24 because no present household member was ≥18 years of age. Of the remaining 2,008 persons, 1,190 (59%) participated in the survey. Of the participants, 718 (60%) reported living in the former West, 462 (39%) in the former East, and 10 (1%) did not report location of residence. Sixty-three percent (452) of survey participants living in the former West and 60% (276) living in the former East were women. The median age was 47 years for persons living in the former West and 51 years for those in the former East. Three percent (33) of survey participants were not German citizens.

Immunization Status

Of the 1,183 participants who reported their vaccination status, 277 (23%) reported having been vaccinated since September 1. Reported immunization rates were much lower in the former West Germany (16%; 115/715) than in the former East (35%; 159/459). The study population differed from the general German population with regard to age structure, gender, and place of residence in the former East or West as reported in the official population data from 1998 (17). The estimated immunization rate for the whole country, adjusted for age, gender, and place of residence (former West or East), was 18% (95% confidence interval [CI] 16% to 21%). The age- and gender-adjusted immunization rate in the former West was 15% (95% CI 13% to 18%), a figure significantly lower than 32% (95% CI 28% to 37%) in the former East.

Sixty-eight percent (489) and 78% (361) of survey respondents living in the former West and East, respectively, reported at least one characteristic that placed them in an influenza immunization target group. Among target group members, reported vaccination rates were nearly twice as high among those living in areas of the former East (40%; 142/358) than those living in areas of the former West (21%; 101/486). Immunization rates were higher among all target subgroups in areas of the former East (Table 1). Vaccination rates were particularly low among health-care workers (7% [95% CI 1% to 13%] in the former West and 10% [95% CI 1% to 19%] in the former East).

Possible Factors Influencing Immunization

We restricted our analyses of factors influencing the immunization rate to those reported by the 587 participants in the target subgroups (aged ≥60 years, chronically ill, and

Table 1. Immunization rates in the target groups for influenza vaccination, former West and East Germany, November 1999

	Former West Germany			Former East Germany		
Target groups	Vaccinated	% (95% CI[a])	Total	Vaccinated	% (95% CI[a])	Total
≥60 yrs of age	78	37 (30% to 43%)	213	90	55 (47% to 63%)	163
Chronic illness	51	31 (24% to 39%)	164	81	49 (41% to 57%)	165
Professional exposure	21	9 (5% to 13%)	243	39	25 (19% to 33%)	155
Health-care workers	4	7 (2% to 16%)	60	2	10 (1% to 30%)	21
Workers with public contact	17	9 (6% to 14%)	183	37	28 (20% to 36%)	134
One or more of the above	101	21 (17% to 25%)	486 [b]	142	40 (35% to 45%)	358[b]

[a]CI, confidence interval.
[b]Totals do not include three persons from former West Germany and three persons from former East Germany for whom information on immunization status was not available.

working in the health-care profession) and for whom information on area of residence in former East or West, as well as vaccination status, was available. Among these persons, those reporting a positive overall attitude towards immunization, those believing that the vaccine is efficacious, and those having had received an influenza vaccination in previous years were much more likely to have been vaccinated in the current immunization period (Table 2). Those reporting fear of contracting influenza from vaccination and fear of other adverse effects had lower immunization rates.

Participants who had read at least one media article in the fall about influenza immunization or who thought influenza a serious illness had similar or slightly higher immunization rates than those without these characteristics (Table 2). Although all the participants included in this analysis reported at least one characteristic of the target subgroups, only 21% (121/587) considered themselves to be at increased risk of contracting influenza compared with the general public.

Role of the Family Physician

Vaccinated survey participants in the key target vaccination groups (those aged ≥60 years, those with chronic illness, and health-care professionals) reported that family physicians performed 93% (84/90) and 94% (106/113) of vaccinations in the former West and East, respectively. Among those participants not vaccinated, more than half (52% in the former West and 63% in the former East) stated that they would have agreed to be vaccinated on the advice of a physician. Persons who reported having a regular family physician had higher immunization rates (Table 2). Those who had visited a physician since September 1, 1999, were more likely to have been immunized than those who had not. During the visit, if the physician had advised immunization, the probability of being immunized increased further. However, only 40% (94/233) and 60% (118/196) of those living in the former West and East, respectively, who reported having had a consultation since September 1, 1999, also reported having been offered influenza vaccination by their physician.

Immunization in the Workplace

Of the working survey participants, 18% (70/386) and 15% (33/228) in the former West and East, respectively, indicated that influenza immunization had been offered at the workplace. For those employed in health-care professions,

Table 2. Factors significantly influencing likelihood of an influenza vaccination during the immunization period (1999–2000) for 587 survey participants, by area of residence, November 1999[a–c]

		Former West Germany				Former East Germany			
		Vaccination				Vaccination			
		Yes	No	OR	95% CI	Yes	No	OR	95% CI
Influenza vaccination in previous years	Yes	69	52	10.4	5.8% to 19.1%	101	42	18.4	8.9% to 40.9%
	No	24	190			12	93		
Positive attitude towards immunization in general	Yes	78	164	7.8	1.9% to 68.9%	108	103	6.3	1.3% to 58.8%
	No	2	33			2	12		
Belief in efficacy of vaccine	Yes	84	159	9.7	2.4% to 85.3%	111	94	12.3	2.9% to 110.9%
	No	2	34			2	21		
Belief that influenza is a severe disease	Yes	73	200	3.1	0.7% to 28.3%	90	106	2.3	0.7% to 10.4%
	No	2	17			4	11		
Information from the media	Yes	48	134	0.9	0.5% to 1.4%	80	77	1.8	1.0% to 3.2%
	No	44	105			32	56		
Regular family physician	Yes	92	217	11.8	1.9% to 490.6%	112	123	5.0	1.1% to 47.2%
	No	1	28			2	11		
Consultation with physician since September 1, 1999	Yes	81	152	4.4	2.2% to 9.6%	109	87	14.6	5.1% to 57.9%
	No	11	91			4	47		
Vaccination offer during consultation[d]	Yes	63	31	13.5	6.7% to 27.9%	83	35	4.7	2.5% to 9.2%
	No	18	121			26	52		
Fear of contracting influenza through vaccination	Yes	17	111	0.2	0.1% to 0.5%	29	62	0.5	0.3% to 0.8%
	No	55	84			60	59		
Fear of adverse effects	Yes	5	54	0.1	0.0% to 0.3%	11	24	0.3	0.1% to 0.7%
	No	80	87			94	62		

[a]OR, odds ratio; CI, confidence interval.
[b]Denominator varies because persons who indicated “don’t know” were not included in the analysis.
[c]Target groups included those ≥60 years of age, the chronically ill, and those who worked as professionals in the health-care sector.
[d]Only persons having seen a physician since September 1, 1999.

these percentages were 35% (21/60) and 43% (9/21) in the former West and East, respectively. Five (6%) of the 80 immunized working participants were immunized at the workplace.

Discussion

We estimate that, as of November 22, 1999, 18% of the German population ≥18 years of age had received influenza vaccination for the 1999–2000 influenza season. This percentage corresponds to the 20% maximum estimate of the immunization rate calculated from the number of vaccine doses sold for the immunization period 1999–2000, assuming all doses sold were given (13.1 million doses for the 1999 influenza vaccination period; data provided by the suppliers). The estimated immunization rate of 18% is substantially lower than the target of 42%, based on percentage of the adult population comprising the key target groups for vaccination. Vaccination rates were nearly twice as high among persons living in the former East than in the former West, despite the fact that the two health-care systems have been unified for almost 10 years. Similar geographic differences in rates existed among all recommended vaccination target groups. Nevertheless, vaccination rates were inadequate among the key target groups of the elderly and those chronically ill in all areas; only approximately one third of persons in the former West and one half in the former East were vaccinated. Another finding was the low vaccination rates among surveyed health-care workers; however, few health-care workers were surveyed.

In Germany, the attitudes and practices of the family physicians may be a critical factor in influencing influenza vaccination rates. Our study showed that persons in key target groups for vaccination (age ≥60 years, chronic illness, health-care professionals) who had had a regular family physician and had had a recent medical consultation during which the physician offered vaccination were much more likely to have been vaccinated. These results are consistent with other studies showing the importance of physicians or health-care personnel in motivating people for influenza vaccination (3,9). Almost all vaccinations, both in the former West and East, were given by a family physician, and over half the nonimmunized participants stated they would have agreed to be vaccinated on advice of a physician. Family physicians thus have a substantial opportunity to improve immunization coverage by more actively and frequently recommending vaccination, especially to persons belonging to a risk group. The fact that 60% of participants belonging to key target groups for influenza vaccination in the former West and 40% in the former East who had seen their physician during the immunization period were not actively offered vaccination indicates many missed opportunities for vaccination. A study by Booth et al. (14) shows that 71% to 82% of general practitioners reported having routinely offered influenza vaccination to patients from risk groups. Although similar data do not exist for Germany, that only 40% to 60% of our survey participants reported having been offered immunization suggests that fewer general practices routinely offer vaccination. Perenboom et al. (18) found that, in the Netherlands, when general practitioners invited their chronically ill patients to be vaccinated, vaccination coverage increased among this group to 75.5%, compared with 42% for the same risk group found in the National Health Interview Survey.

Health-care workers represent a specific target group with an extremely low vaccination rate. Because the number of health-care workers who participated in this study was low (81 workers), results must be carefully interpreted. Our results, however, were confirmed by later studies (Hallauer et al., unpub. data; 6). Health-care workers might not be reached by family physicians, but rather by alternative interventions such as vaccination programs at the workplace. However, this group needs further investigation regarding targeted interventions to increase vaccination coverage.

Among those in key target groups for vaccination, persons who had received an influenza immunization in a previous year were much more likely to have been immunized during the current period. This conclusion is consistent with the findings of several previous studies (19–28). Therefore, a concerted effort to increase vaccination coverage in target groups in 1 year might have a positive impact on revaccination in the following years. This success rate might be one reason for the persistently higher vaccination rates among persons living in the former East.

Our results suggest several possibilities for improving influenza vaccination rates in Germany. One possibility would be to better focus the target populations for influenza vaccination. We found that approximately 70% of the population fit into a target vaccination group, largely due to the category comprising public exposure in the workplace. Despite the fact that our definition for this target group (persons who spend more than half their working day dealing with many people not their colleagues) was narrower than that used by the German Standing Commission on Immunization (public exposure in the workplace), this group included almost half the participants belonging to target groups. Were this group defined more precisely, the criteria could be communicated more clearly to family physicians and employers and thus make the indications for immunization less ambiguous.

Fear of contracting influenza through immunization and fear of adverse effects had a negative impact on the immunization rate, as seen in other studies (3,9,19,21,22,24,26,28,29–33). Health information messages, particularly those given by physicians aimed at reducing these fears, may have a beneficial effect on vaccination rates. Earlier research suggests that the self-perception of influenza risk is often inaccurate (1,2). Our study confirms this finding; only about one fifth of those participants belonging to a target group were, in their own opinion, at higher risk of becoming more severely ill from influenza than the general population. Again, we suggest that targeted information about risk factors for influenza and complications should be enforced. Another way to increase vaccination rates may be to improve workplace immunization programs, particularly for health-care workers. We found that <40% of health-care workers interviewed in this study

reported having a workplace influenza immunization program. With <10% of the health-care workers reporting having been vaccinated, the existing programs must be largely ineffective.

Our study has several limitations. The study population differed from the general population in age, gender, and place of residence. To avoid possible biases because of these differences, we used standardized figures. Influenza vaccination was self-reported; because the survey was anonymous, confirmation of vaccination status was not possible. In addition, because we were unable to repeatedly call households on different days if nobody answered the phone on the first try, persons who spent more time at home were probably more likely to participate, resulting in an overrepresentation of persons in certain vaccination target groups (age ≥60 years, chronic illness). Persons who lived in households without telephones or could not speak German were also not sampled. If these groups have a lower vaccination rate, our estimated vaccination rate will then have been overestimated.

The timing of the survey (late November) may have led to an underestimate of the true vaccination rate because participants might have been vaccinated later in the season. No studies from Germany on vaccine uptake during the vaccination period have been available up to now. However, the overall vaccination rate shows that the study (18%) and the vaccination rate calculated by using the number of doses sold (20%) correspond closely and suggests that the number of persons vaccinated in the later months of the immunization period was low. Although the German influenza vaccination experience reported in this study suggests areas for improvement, the circumstances resulting from the German reunification also demonstrate the long-term benefits of a sustained, concerted effort to improve influenza vaccination rates.

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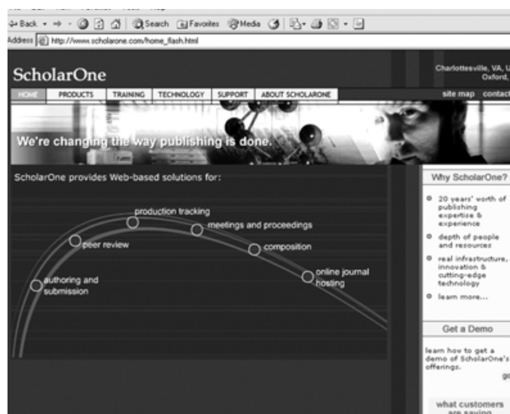

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Legionnaires' Disease at a Dutch Flower Show: Prognostic Factors and Impact of Therapy

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After a large outbreak of Legionnaires' disease in the Netherlands, we determined risk factors for intensive care unit (ICU) admission and death and the impact of adequate therapy on ICU-free survival among 141 hospitalized patients. Overall mortality rate was 13%, and ICU mortality rate was 36%. Smoking, temperature >38.5°C, and bilateral infiltrates shown on chest x-ray were independent risk factors for ICU admission or death (all $p<0.05$). Starting adequate therapy within 24 hours after admission resulted in a higher ICU-free survival rate compared to therapy initiation after 24 hours: 78% versus 54%, respectively ($p=0.005$). However, delay in providing therapy to patients with urinary antigen tests with negative results did not influence outcome. These data suggest that by using the urinary antigen test on admission a more tailored approach to patients with community-acquired pneumonia may be applied.

Severe Legionnaires' disease has an overall mortality rate of 10% to 30% (1–3), and 30% to 50% of patients require admission to an intensive care unit (ICU) (1,4). One of the most important determinants of outcome is the early initiation of adequate therapy after admission (1,5). Administering appropriate antibiotics for *Legionella pneumophila* during the empiric treatment of patients with community acquired pneumonia has been advocated (6). Given the low frequency of Legionnaires' disease, this strategy is costly and leads to overconsumption of antibiotics. Therefore, many physicians have not adopted these guidelines in daily practice. Identifying those patients with community-acquired pneumonia caused by *L. pneumophila* is difficult.

In March 1999 one of the largest outbreaks of Legionnaires' disease since the first described outbreak in Philadelphia (7) occurred in the Netherlands. The outbreak originated at the Westfriesian Flora, an annual flower show combined with a consumer products exhibition, held February 19–February 28, 1999. The flower show was visited by 77,061 persons, and Legionnaires' disease developed in at least 188 (8). The size of the outbreak provided a unique opportunity to determine which clinical factors on hospital admission predict ICU admission or death (ICU/death). We also evaluated whether the rapid urinary antigen test can help identify those patients with Legionnaires' disease for whom adequate therapy cannot be delayed.

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Patients and Methods

Study Group

On March 12, 1999, the Dutch population was alerted by newspapers and a special broadcast that a flower show was identified as probable origin of an outbreak of Legionnaires' disease (9). To collect clinical data, we obtained written informed consent from patients with Legionnaires' disease who had visited the flower show or their relatives. The study was approved by the medical ethical committee of the Academic Medical Center in Amsterdam.

The following definitions were used to categorize the patients:"confirmed Legionnaires' disease" was defined as the presence of a new infiltrate shown on the chest x-ray on admission and one or more of the following laboratory criteria: 1) isolation of *L. pneumophila* from a respiratory sample (28 patients), 2) detection of *L. pneumophila* serogroup 1 antigen in a urine sample (Binax Now *Legionella* urinary antigen test; Binax, Portland, ME) (86 patients), 3) seroconversion to positive immunoglobulin (Ig)G or IgM (or both) antibody levels to *L. pneumophila*, or a fourfold rise in antibody titers to *L. pneumophila* in paired acute-phase and convalescent-phase sera (62 patients). Antibodies to *L. pneumophila* were determined by using a commercial enzyme linked immunosorbent assay (IgM cutoff >140 U/mL and IgG cutoff > 70 U/mL, serogroup 1–7; Serion, Institut Virion-serion GmbH, Wurzburg, Germany) or a microagglutination antibody assay (IgM, serogroup 1-12; Regional Laboratory of Public Health, Tilburg, the Netherlands). In three patients, a commercial enzyme immunoassay (Binax EIA, Binax; Biotest EIA, Biotest AG, Dreiech, Germany) was positive in concentrated urine, while other diagnostic tests were negative.

"Probable Legionnaires' disease" was defined as the presence of a new infiltrate shown on the chest x-ray on admission and either a single high antibody titer (microagglutination assay 1:>256; 2 patients) or a positive polymerase chain reaction analysis of sputum (1 patient) (10). Patients who visited the flower show and in whom radiologically confirmed pneumonia developed within 4 weeks were also considered to have probable Legionnaires' disease when no other cause of the pneumonia could be established (18 patients). Patients were excluded if the first symptoms occurred more than 4 weeks after they visited the flower show. None of the patients were hospitalized during the month preceding admission.

Data Collection and Definitions

Data on the following variables were collected from the medical chart (if data were missing, patients were interviewed by telephone): ,1) Premorbid conditions: age, sex, smoking $\geq$1 cigarette per day), alcohol intake ($\geq$2 units per day), use of immunosuppressive medication (ongoing treatment with chemotherapy or steroids >10 mg/day), underlying diseases such as chronic obstructive pulmonary disease, diabetes mellitus, chronic renal insufficiency, cancer (solid or hematologic neoplasm), and chronic cardiac disease (considered present if cardiac medication was used at the time of the flower show visit). 2) Day of visiting the flower show, first day of illness, and date of admission 3) Symptoms and results of physical examination on admission. 4) Routine biochemical and hematologic laboratory tests obtained on admission. 4) Urinary antigen test results collected from the microbiologic laboratory that performed the test. The overall agreement between the Binax NOW and the enzyme immunoassay Binax EIA (Binax *Legionella* Urinary Antigen EIA Kit: Binax) has been found to be 98% (11). 6) Chest radiograph results on admission and 48–72 hours later reviewed by attending hospital radiologist. Radiographic progression during this period was defined as an increase in density or size of infiltrate, or progression to multiple lobes. 7) Antibiotic treatment. Adequate therapy was considered a macrolide or a fluoroquinolone, with or without rifampicin. 8) Admission to the ICU, death, and renal insufficiency.

At the time of the patient's admission, Legionnaires' disease was defined as severe when two or more of the following conditions were present: 1) respiratory rate >30 breaths / minute, 2) chest radiograph showing bilateral involvement or involvement of multiple lobes, 3) shock (systolic blood pressure below 90 mmHg or diastolic blood pressure below 60 mmHg), 4) PaO_2 <60 mmHg or arterial oxygen saturation <92%. For assessment of severity, we used the minor criteria for severity of community-acquired pneumonia described by the American Thoracic Society (12) since the major criteria are indicators for ICU admission by themselves.

Statistical Analysis

The independent relation between clinical factors and the dependent variable, ICU admission or death (whatever came first), were assessed with univariate and multivariate logistic-regression models. Factors with a p value >0.20 in the multivariate analysis were excluded from the final multivariate analysis. Continuous variables were compared using a t test for groups; categorical variables were compared by using the chi-square test. A two-tailed p value of 0.05 or less was considered to indicate statistical significance.

Kaplan Meier survival analysis was used to compare the ICU-free survival between patients in whom adequate therapy was initiated within or later than 24 hours after admission. ICU-free survival was defined as survival without admission to the ICU during hospitalization.

Results

Patients

Of 188 identified patients with confirmed or probable Legionnaires' disease during the outbreak (8), 161 patients gave permission to collect clinical data (Figure 1). Since severity of illness did not warrant hospital admission in 20 patients and limited clinical, laboratory and radiologic information was available for these 20 patients, they were not included in the final analysis. Among these 20 patients were 15 confirmed and 5 probable cases; none of these patients died during the course of Legionnaires' disease. The final analysis was done on 141 hospitalized patients.

Forty-two (30%) of these 141 patients were admitted to the ICU, 40 (95%) of whom had confirmed Legionnaires' disease.

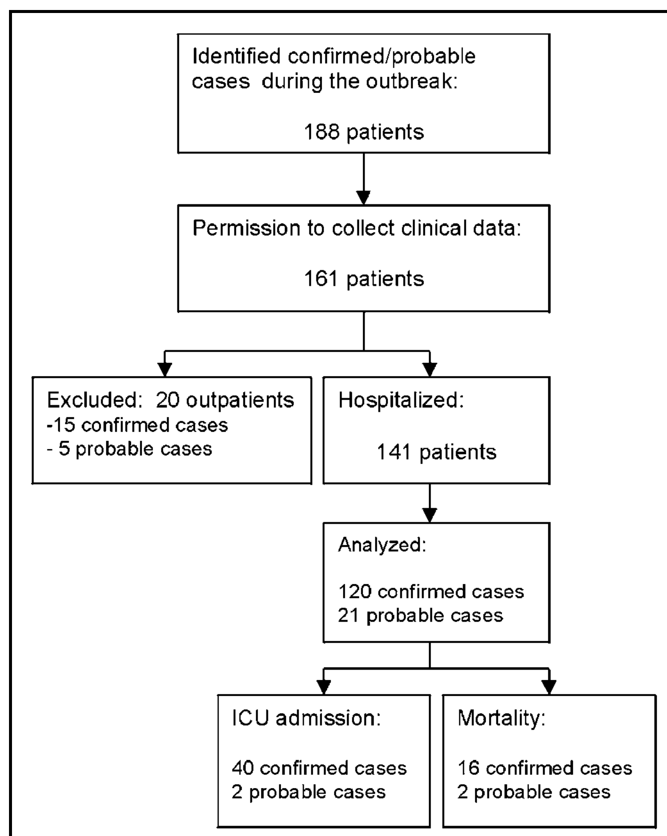

Figure 1. Patient disposition and selection. ICU, intensive care unit.

Overall mortality rate was 13%, and ICU mortality rate was 36% (Figure 1). The median incubation time was 7 days (range 1–18 days). The incubation time did not significantly differ between patients with severe Legionnaires' disease (mean 6.8 days, [SD 3.5]) and those with nonsevere pneumonia (mean 7.8 days [SD 3.1]: t test p=0.13) or between patients who were admitted to the ICU or died (mean 7.0 days, [SD 3.1]) and those who did not (mean 7.7 days, [SD 3.5]: t test p=0.26).

The nationwide alert on March 12 led to an increase in hospital admissions. Patients admitted after the alert (n=71) were less severely ill: 21% had severe Legionnaires' disease in contrast to 44% before the alert (n=70). As expected, patients with severe Legionnaires' disease (46/141, 33%) had an increased risk for ICU admission or death compared with nonseverely ill patients (OR 4.5, CI 2.1 to 9.6), p=0.001).

Characteristics on Admission

The patients' clinical, laboratory, and radiologic data on admission are shown in Table 1. The median age was 67 years (range 21–92 years) and more were male (58%). Eighty-eight (62%) patients had at least one underlying disease; cardiac disease was the most common. In the univariate analysis, smoking, dyspnea, fever above 38.5°C, plasma creatinine level >100 mmol/L, and bilateral infiltrates or pleural effusions shown on the chest x-ray at admission were found to predict subsequent ICU admission or death (Table 1). In the multivariate analysis, smoking, temperature >38.5°C, and bilateral infiltrates on admission were independent risk factors for ICU admission or death (Table 2).

During hospitalization, lung infiltrates shown on the chest x-ray progressed within 24–48 hours in 40% of the patients. This progression was not associated with ICU admission or death. In 39 patients (35%), renal insufficiency developed during admission (serum creatinine level above 130 mmol/L at any time during admission). Development of renal insufficiency was associated with ICU admission or death (OR 5.4, CI 2.3 to 12.7). None of the patients who survived had persistent renal insufficiency. In this large group of patients with Legionnaires' disease, no other symptoms suggested extrapulmonary foci of infection.

Therapy and Delay in Therapy

Of the 70 patients admitted before the nationwide alert on March 12, 44 (63%) were treated with adequate antibiotics with a median delay of 1.5 days (range 0–14 days). After the alert, antibiotics were changed to a macrolide or a fluoroquinolone for 21 patients, and 5 patients were never treated with adequate antibiotics (three of them died). All patients admitted after the alert received adequate therapy within a median of 0 days (range 0–3 days). Next, we studied the influence of immediate start of adequate treatment compared with delayed treatment on the outcome. Initiation of adequate therapy within 24 hours after admission resulted in a higher ICU-free survival rate compared with initiation after 24 hours: 78% versus 54% (Figure 2;

Table 1. Univariate analysis of factors determining outcome[a]

	No. patients (%)	Odds ratio (95% CI)[b]	p value
Patient characteristics			
Male	82 (58)	1.5 (0.7 to 3.1)	0.30
Age >67 years	75 (53)	1.0 (0.5 to 2.1)	0.98
Underlying diseases			
COPD	11 (8)	0.8 (0.2 to 3.1)	0.73
Diabetes mellitus	16 (11)	1.0 (0.3 to 3.0)	0.95
Renal insufficiency	3 (2)	1.1 (0.1 to 12.1)	0.96
Cardiac disease	48 (34)	1.0 (0.5 to 2.0)	0.90
Cancer	10 (7)	0.5 (0.1 to 2.5)	0.41
Immunosuppressive medication[d]	11 (8)	1.9 (0.5 to 6.5)	0.52
Smoking[c]	65 (48)	2.4 (1.2 to 5.1)	0.02
Alcohol intake[c]	26 (59)	3.7 (0.8 to 15.8)	0.08
Symptoms			
Fever	119 (84)	0.8 (0.3 to 2.0)	0.63
Myalgia	31 (22)	0.6 (0.2 to 1.4)	0.21
Headache	36 (26)	0.6 (0.3 to 1.5)	0.30
Cough	97 (69)	1.0 (0.5 to 2.2)	0.99
Dyspnea	79 (56)	2.6 (1.2 to 5.5)	0.01
Diarrhea	25 (18)	1.3 (0.5 to 3.1)	0.63
Confusion	31 (22)	1.8 (0.8 to 4.0)	0.18
Physical examination			
Temperature >38.5°C	101 (72)	3.6 (1.4 to 9.3)	0.009
Respiratory rate >18/min[c]	34 (85)	5.6 (0.6 to 53.4)	0
Biochemistry[c]			
Sodium <130 mmol/L	36 (26)	2.1 (1.1 to 4.7)	0.06
Creatinine >100 µmol/L	73 (52)	2.1 (1.0 to 4.4)	0.05
CPK >200 U/L	25 (50)	1.4 (0.5 to 4.2)	0.57
ASAT >100 U/L	21 (18)	1.7 (0.6 to 4.4)	0.30
γ-GT >100 U/L	12 (13)	0.42 (0.09 to 2.03)	0.28
PO_2 <9.7 kPa	96 (83)	0.64 (0.24 to 1.70)	0.37
X-ray results			
Bilateral infiltrates[e]	38 (27)	3.5 (1.6 to 7.6)	0.002
Pleural effusion	15 (11)	3.8 (1.2 to 11.3)	0.002
Progression within 48 hrs[c,f]	46 (41)	1.6 (0.7 to 3.4)	0.25

[a]Logistic regression analysis.
[b]CI: confidence interval, COPD, chronic obstructive pulmonary disease, CPK, creatinine phosphokinase; ASAT, aspartate aminotransferase; g-GT, gamma glutamyltransferase.
[c]Data for smoking (≥1 cigarette per day), alcohol intake (≥2 U per day), breathing frequencies, laboratory tests, and progression of infiltrates were available for a proportion of patients. Cutoff levels for CPK, ASAT, and α-GT are two times the upper normal limit.
[d]Immunosuppressive medication is defined as ongoing treatment with chemotherapy or steroids >10 mg/day.
[e]Unilateral infiltrate was the reference group.
[f]Radiographic progression during 24–48 hours was defined as an increase in density or size of the infiltrate, or progression to multiple lobes.

Table 2. Multivariate analysis of factors determining outcome

Prognostic factor[a]	Odds ratio (95% CI)[b] (n=141)	p value
Smoking	2.5 (1.1 to 5.6)	0.03
Dyspnea at presentation	2.1 (0.9 to 4.8)	0.09
Temperature >38.5°C	2.9 (1.0 to 8.6)	0.05
Plasma creatinine >100 µmol/L	2.0 (0.9 to 4.6)	0.11
Bilateral infiltrates	4.2 (1.7 to 10.3)	0.002
Pleural effusion	3.4 (0.99 to 11.6)	0.053

[a]Prognostic factors with p≤ 0.05 in the univariate analysis were entered. Factors with p value >0.2 in the multivariate analysis were excluded.
[b]CI, confidence interval.

log rank: p= 0.005). The difference in ICU-free survival was not explained by differences in severity of pneumonia in the two groups, since the percentage of patients with severe pneumonia in the group treated within 24 hours (31% severe Legionnaires' disease) did not significantly differ from the percentage in the group adequately treated after 24 hours (36% severe Legionnaires' disease; chi square: p=0.5).

A Binax Now urinary antigen test with positive results can provide a diagnosis of *Legionella* pneumonia within 1 hour. This test was positive in 86/141 (61%), negative in 51/141 (36%) and not done in 4/141 (3%) of the patients. Table 3 shows the results of other diagnostic tests of patients with positive and negative urinary antigen test results. In 16 patients with negative urinary antigen test results, no other test had positive results, although the clinical and epidemiologic features strongly suggested Legionnaires' disease. Two of these patients were admitted to the ICU. Patients with negative urinary antigen test results had a higher ICU-free survival rate than patients with positive test results: 90% ICU-free survival compared with 58% of those with positive test results (Figure 3A; log rank: p=0.0001). No effect on outcome was found when initiation of adequate therapy was delayed in patients with a negative urinary antigen test (Figure 3B, 92% vs. 84% ICU-free survival; log rank: p= 0.46). In contrast, patients with positive urinary antigen test results in whom adequate therapy was started within 24 hours after admission had a higher ICU-free survival rate compared with patients in whom therapy was initiated after 24 hours (67% vs. 48% , Figure 3C; log rank: p= 0.09), resulting in a relative risk reduction of 38%. The proportion of patients with severe pneumonia was comparable for both groups of patients with a positive urinary antigen test (within 24 hours: 39%, after 24 hours, 45% severe Legionnaires' disease, chi square: p= 0.58).

In addition, 36 (38%) of 95 patients with nonsevere Legionnaires' disease were treated with adequate antibiotic therapy >24 hours after admission; 13 of those patients (36%) had a poor outcome. In 10 (77%) of these patients, the urinary antigen test was positive for *L. pneumophila*, indicating that these patients should have been identified as high risk on admission.

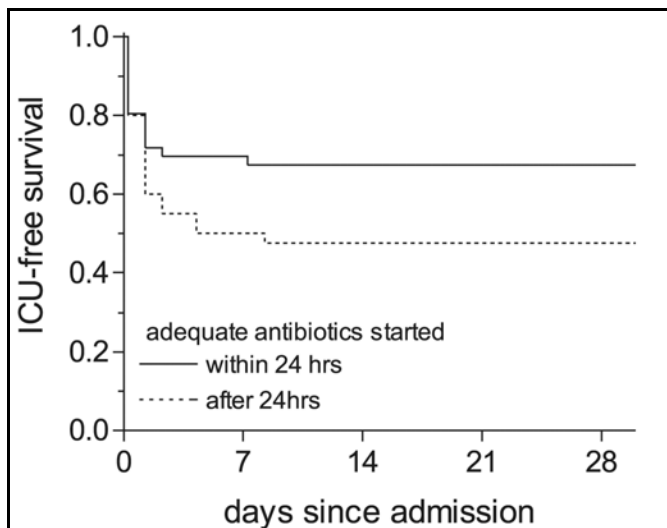

Figure 2. Kaplan-Meier curve for intensive care unit (ICU)–free survival. ICU-free survival for patients treated with adequate antibiotics within and >24 h after admission.:___ adequate antibiotic therapy started within 24 h after admission (n=85); ----- adequate antibiotic therapy started >24 h after admission (n=56).

Discussion

Since the first outbreak of Legionnaires' disease in Philadelphia in 1976 (7), several outbreaks have been described that were linked to hospitals, hotels, cooling-towers, and whirlpool baths (13–16). The outbreak reported here is the largest outbreak associated with a contaminated whirlpool spa located at the exhibition hall of a flower exhibition. Analysis of 141 hospitalized patients showed that a history of smoking, fever >38.5°C, and bilateral infiltrates shown on chest x-ray were associated with an increased risk for ICU admission or death. A urinary antigen test with positive results was also associated with poor outcome. Initiation of adequate therapy within 24 hours after admission showed a higher ICU-free survival rate compared to initiation of therapy after 24 hours. No protective effect of early adequate therapy was found in patients with Legionnaires' disease and a urinary antigen test negative for *L.*

Table 3. Positive results of other diagnostic tests of patients with positive and negative urinary antigen tests[a]

Diagnostic test	Positive urinary antigen test (n=86)	Negative urinary antigen test (n=51)
Sputum culture	23	3
Fourfold rise in titer/ seroconversion	35	27
Polymerase chain reaction	9	1
Single high titer	5	3
Positive urinary antigen ELISA[b] test in concentrated urine[c]	NA	7
No positive test results	29	17

[a]Urinary antigen test results were based on the test result of the qualitative immunochromatographic assay (Binax Now, Binax, Portland, ME).
[b]ELISA, enzyme-linked immunosorbent assay; NA, not applicable.
[c]Positive Binax EIA and Biotest EIA in concentrated urine samples.

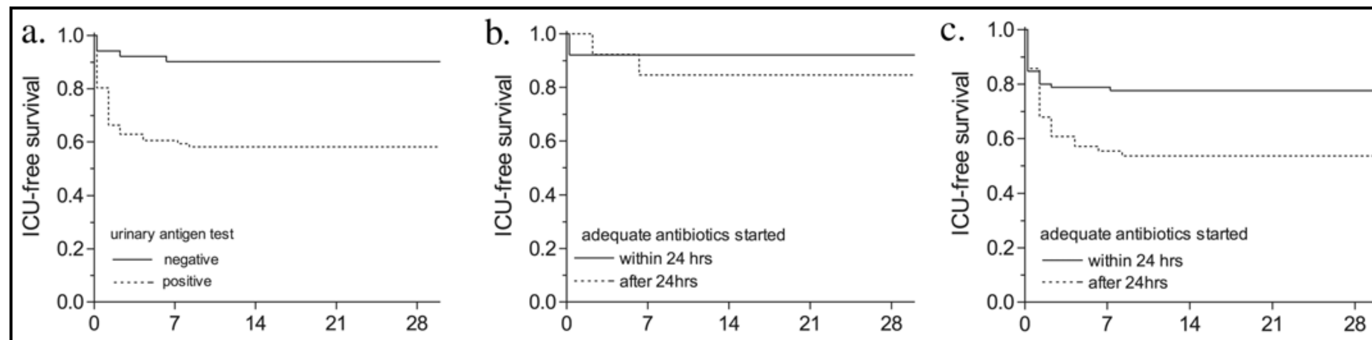

Figure 3. Survival curves and urinary antigen test results. A: Intensive care unit ICU)–free survival for patients with a positive or negative urinary *Legionella* antigen test (Binax Now, Binax, Portland, ME):___ negative urinary antigen test (n=51); ----- positive urinary antigen test (n=86). B: ICU-free survival for patients with a negative urinary *Legionella* antigen test (Binax Now):___ adequate antibiotic therapy started within 24 h after admission (n=38); ----- adequate antibiotic therapy started more than 24 h after admission (n=13). C: ICU-free survival for patients with a positive urinary *Legionella* antigen test (Binax Now): ___ adequate antibiotic therapy started within 24 h after admission (n=.46); ----- adequate antibiotic therapy started >24 h after admission (n=40).

pneumophila. However, in patients with positive urinary test results, early adequate therapy reduced the risk of ICU admission and death by 38%.

The endpoint of either ICU admission or death was chosen because only 18 patients died, which strongly decreased the power of the analysis. In clinical practice, preventing ICU admission with all the disadvantages of such an admission in terms of sickness and death, is one of the goals of early treatment. Since >80% of the diseased patients were first admitted to the ICU, we chose to combine ICU admission and death as a composite primary outcome parameter.

We analyzed 141 hospitalized patients and excluded 20 outpatients. However, this group represents only 20 out of 161 patients, and the described 141 patients represent 88% of all patients. In a study by Boshuizen et al. (17), a survey among the 700 exhibitors at the flower show revealed no symptomatic infections (for example, Pontiac fever); these researachers concluded that either pneumonia develops in exposed persons or they remain healthy. Therefore, this study elucidates the complete range of the severity of the pneumonia that develops in these patients.

The case definition for probable cases was broad enough to ensure inclusion of patients who died before the diagnostic work-up for *Legionella* was completed. Of the 21 probable case-patients, 18 had no single diagnostic test with positive results and showed no evidence of infection by other microorganisms (4 ICU admissions of which 2 died). Despite clinical and epidemiologic features suggestive of Legionnaires' disease, other undetected causes of pneumonia cannot be excluded.

Patients with Legionnaires' disease are more likely to have severe pneumonia requiring ICU admission than are patients with community-acquired pneumonia caused by other organisms (1,18,19). In this study, 42 (30%) patients) were admitted to the ICU. The overall mortality rate (13%) and ICU mortality rate (36%) in our patients were consistent with earlier reports (1,3,4).

For all patients, the exposure day and the date when first symptoms occurred were known. The incubation time ranged from 1 to 18 days, which is longer than the upper limit of 12 days reported previously (7,20). The virulence of the causative *Legionella* strain, as assessed by its potential for intracellular growth (21), did not differ from that of other clinical isolates and cannot account for this long incubation time. This longer incubation means that Legionnaires' disease can no longer be excluded as a potential cause of community-acquired pneumonia when, for example, the person traveled >12 days ago.

Male gender, older age, underlying diseases like chronic obstructive pulmonary disease, diabetes mellitus, and immunosuppressive medication, reported by others as predictors for fatal outcome (2–4), were not associated with poor outcome in this study, although the prevalence of underlying diseases in our population was similar to the prevalence in earlier studies describing community-acquired Legionnaires' disease. This difference from other studies might be explained by the fact that in our study no selection was made for ICU patients, and no patients with nosocomial disease were included (2–4,22).

Patients who sought treatment with bilateral infiltrates (27%) and with pleural effusion (11%) had an increased risk for ICU admission or death. In a prospective study on chest radiographic findings in patients with community-acquired Legionnaires' disease, 16% of the patients had bilateral involvement, and 23% had pleural effusions on admission, which increased to 30% and 63%, respectively, during hospitalization (23). Despite some lung deterioration, which was visible on chest x-ray, most patients improved clinically. In this study, progressive lung deterioration within 48–72 hours (noted in 41% of the patients) was not a significant risk factor for ICU admission or death. Bilateral involvement on admission, on the other hand, was the most powerful prognostic factor associated with poor outcome in the multivariate analysis.

Identification of patients with Legionnaires' disease has important implications for the choice of initial therapy. Studies comparing the clinical manifestations of *Legionella* pneumonia to other types of pneumonia have indicated that Legionnaires' disease is not "atypical" and that individual clinical features such as diarrhea, confusion, hyponatremia, and chest x-ray findings are not sufficiently distinctive to distinguish Legionnaires' disease from other types of community-acquired pneumonia (18,24–26). The results of cultures

require several days, and serum antibody tests have a low sensitivity. In patients with Legionnaires' disease related to this outbreak, sensitivity was approximately 43% for one of the three separate antibody tests and 61% for any of the tests, using a positive culture result, a urinary antigen test with positive results, or both as the criterion standard (E. Yzerman, pers. comm.).

Detection of *L. pneumophila* antigens in a urine sample provides a diagnosis within 1 hour, with a specificity of 95% to 100% (11) (Binax Now *Legionella* urinary antigen test, Binax). Patients in our study with a positive urine test during hospitalization had an increased risk for ICU admission or death, in accordance with data indicating that the percentage of positive test results increased with the clinical severity of the disease (27). Although the urinary antigen test was done retrospectively in many patients (median 9 days after the first symptoms, range 0–25 days), the number of positive urinary tests is not lower during the first 3 days after symptoms than after 3 weeks of illness (28). The urinary antigen test used during this outbreak detects *L. pneumophila* serogroup 1, which is responsible for approximately 70%–80% of Legionnaires' disease cases in the United States and Europe.

Increased deaths associated with delay of adequate treatment for Legionnaires' disease has been reported earlier (1,5); in patients suspected of having Legionnaires' disease, adequate therapy should therefore be started as soon as possible. To ensure coverage of potential

L. pneumophila infections in every patient, the recommendations for treatment of patients with community-acquired pneumonia have been expanded. The new guidelines from the Infectious Diseases Society of America recommend an extended-spectrum cephalosporin plus a macrolide or a fluoroquinolone alone, for every hospitalized patient in whom no pathogen is defined (6). This approach may lead to overtreatment since 2%–13% of community-acquired pneumonia is caused by *L. pneumophila* (18,29,30). Therefore, this approach is costly and, in addition, may contribute to macrolide and fluoroquinolone resistance.

The results of our study suggest that a more tailored approach of patients with community-acquired pneumonia may be possible. When Legionnaires' disease is considered in the differential diagnosis of patients with community-acquired pneumonia, a urinary antigen test should be done on admission. If test results are positive, the patient should be treated immediately with a fluoroquinolone or a macrolide since a positive urinary test on admission identifies the patients with Legionnaires' disease caused by *L. pneumophila* serogroup 1 and a high risk for ICU admission or death. If the urinary antigen test gives negative results, deferring anti-*Legionella* therapy for the first 24 hours after admission, pending the diagnostic work-up, may be justified because the outcome in Legionnaires' disease is not influenced. In this way, unnecessary use of antibiotics in patients hospitalized with community- acquired pneumonia may be avoided.

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Leptospirosis: Skin Wounds and Control Strategies, Thailand, 1999

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After an outbreak of leptospirosis in workers who participated in cleaning a pond during September 1999 in Thailand, a serologic survey was conducted. Among a cohort of 104 persons from one village who participated in pond cleaning activity, 43 (41.3%) were seropositive for immunoglobulin M antibodies against *Leptospira*, indicating recent infection. Only 17 (39.5%) of 43 seropositive persons reported a recent febrile illness; the remaining seropositive persons were considered asymptomatic, suggesting that asymptomatic leptospirosis infection may be common where leptospirosis is endemic. Multivariable logistic regression indicated that wearing long pants or skirts was independently protective against leptospirosis infection ($OR_{adjusted}$ = 0.217), while the presence of more than two wounds on the body was independently associated with infection ($OR_{adjusted}$ = 3.97). Educational efforts should be enhanced in areas where leptospirosis is endemic to encourage the use of protective clothing. In addition, wound management and avoidance of potentially contaminated water when skin wounds are present should be included in health education programs.

Leptospirosis, a worldwide zoonotic disease, is caused by spirochetes of the genus *Leptospira*. In Thailand, a nationwide leptospirosis epidemic is ongoing and control strategies are being explored (1–5). The number of cases reported from 1982 to 1995 ranged from 55 to 272 cases per year, with an average incidence of 0.3/100,000/year (5). The number of leptospirosis cases reported in 1996 was 398 (incidence 0.65/100,000); the number of cases in 1997 was 2,334 (3.83/100,000). In 1998, the number of cases was 2,230 (3.52100,000), in 1999, the number of cases was 6,080 (9.89/100,000), and in 2000, the number of cases was 14,286 (23.2//100,000) (6). In Thailand, leptospirosis corresponds with the rainy season, with an increase in cases beginning in August and decreasing in November; the peak number of cases occurs in October (6).

Surveillance data suggest that most infections occur in agricultural workers, primarily rice producers (1–5). Infection in humans occurs through contact of skin or mucous membranes with water or moist soil contaminated with urine of infected animals (7–10). Breaks in skin in facilitate infection, but no previous study has quantified the correlation between skin wounds and leptospirosis (11,12). Heavy rainfall and flooding; going without shoes; washing in streams; and occupations such as farming, working in sewers, mining, working with animals, and participating in military activities have all been implicated in human infection (7,10,13–19). Despite identification of these risk factors, control strategies for leptospirosis are lacking.

In September and October 1999, an outbreak of leptospirosis occurred in the Khumuang subdistrict of Buriram, a province in northeastern region of Thailand. No cases of leptospirosis had been reported in this subdistrict for the previous 2 years. Local health officers from the Khumuang Hospital notified the Khumuang District Health Office of an abnormal increase in numbers of patients meeting the World Health Organization (WHO) criteria for leptospirosis infection; 80 cases of leptospirosis were identified from September 19 to 29, 1999.

In association with the outbreak, we conducted a study of persons who participated in pond cleaning activities and used a nested case-control study to compare participants with and without leptospiosis infection. Pond cleaning activities included entering the water, pulling up foliage, and removing debris. The objectives of the study were to 1) estimate the attack rate among pond cleaners in this setting, 2) determine risk factors for leptospirosis infection, and 3) identify possible control and prevention strategies. The results of this study, including the attack rate of leptospirosis, the association between modifiable risk factors for leptospirosis and infection, and the prevalence of asymptomatic infection are reported.

Materials and Methods

Study Design and Method

A cross-sectional survey of pond cleaning participants was conducted from October 6 to 8, 1999, in the subdistrict of

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Khumuang, Buriram Province, Thailand. The survey was conducted with a convenience sample of 315 persons from the total number who participated in the pond cleaning (n=500). All 315 persons were part of an agricultural community located in the Khumuang subdistrict, approximately 400 km from Bangkok and 100 km from Thai-Cambodian border.

Twelve teams, each consisting of three or four health-care providers (doctor or nurse, health officer, and health volunteer), visited this village from October 6 to 8, 1999. All 315 persons from the village involved in the cleaning of Nong Tad were asked to participate in this study. Two hundred twenty-eight (72%) workers consented to be interviewed and were given a standardized questionnaire. Questions included demographics and risk factor information, such as the working site in the pond, lunch eating site, clothing worn while working, other probable exposure to leptospirosis, and presence of wounds during their participation in the cleaning activity. If a participant answered yes to having skin wounds during the pond cleaning activity, his or her body was examined for lesions or scars. and the interviewer subsequently noted the affected body parts. Participants were asked if they had been ill since participating in the pond cleaning activity. Illness was defined as having symptoms meeting the WHO criteria for leptospirosis (20). Clinical information was collected through review of medical records for those participants who reported being ill and who were seen at the Khumuang Hospital. Serum samples were obtained 1 week after the interview from 104 (45.6%) of the 228 participants; the other 124 persons refused to undergo phlebotomy. Therefore, the interviewer did not know the seropositivity status of the participants at the time of the interview. The timing of the serum collections was within 1 month after exposure to the pond. Infected and noninfected pond cleaning participants were evaluated for risk factors for infection in a nested case-control study. All specimens were tested in the Thailand Ministry of Health Laboratories by using the Lepto-Dipstick Test (Organon, Dublin, Ireland), a commercial test kit with sensitivity and specificity exceeding 80% (21). This study was determined to be a public health response that did not require IRB review.

Case Definition

A case was defined as the presence of immunoglobulin (Ig) M anti-leptospiral antibodies by the Lepto-Dipstick Test in a person from the Khumuang subdistrict who participated in the survey. Persons with a positive IgM antibody response were considered to have incident cases because the serum was collected and tested 1 month after exposure and IgM antibodies last an average of 3–6 months (22–24). Asymptomatic infection was defined as a positive IgM response in a person who did not report having fever, myalgias, headache, or other evidence of leptospirosis.

Statistical Analysis

Descriptive statistics and subsequent multivariable analysis were derived through the use of SAS software release 8.1 (SAS Institute, Inc., Cary, NC). All variables were dichotomized except for age, which was treated as a continuous variable.

Demographic, environmental, and behavioral exposure variables were compared for infected and noninfected persons by univariate analyses. All two-way interactions between variables were tested. A multivariable model was created by inclusion of all exposures significant by univariate analysis as well as age and sex to control for confounding, and a backward elimination procedure was performed to identify exposure variables most strongly associated with seropositivity for leptospirosis infection. Confounding in the absence of interaction was assessed by comparing odds ratios (OR) of the exposure variables in the gold standard model controlling for the covariates, age and sex, with the odds ratios of the exposure variables in the reduced models without age and sex, respectively. If a difference of >10% between the OR was detected, confounding was present and the covariate was retained in the model (25). The variance-covariance matrix allowed for the calculation of 95% confidence intervals (CI) for the OR involving the estimated coefficient of any significant interaction term. A SAS macro was used to calculate the conditional indices and variance decomposition proportions, allowing for the assessment of multicolinearity for two or more variables.

Results

Blood samples were collected and tested from 104 (45.6%) of 228 pond cleaning participants for serologic testing. The subset of 104 persons who agreed to participate in the serosurvey was similar to nonparticipants in the distribution of age (p=0.387) and sex (p=0.124). In addition, all 228 participants reported farming as their occupation. The serologic survey population consisted of 55 men and 49 women with a median age of 38.5 years (range 15–65).

Of the 104 serum samples tested by the Lepto-Dipstick Test, 43 were seropositive for IgM antibodies against *Leptospira*, indicating recent leptospirosis infection (attack rate=41.3%).

Infection Attack Rate and Asymptomatic Infection

Of the 43 persons with IgM anti-*Leptospira* antibodies, only 17 (39.5%) reported having illness that met the WHO criteria for leptospirosis; the remaining 26 (60.5%) had asymptomatic infection. Clinical information was available for 13 (76.5%) of the 17 infected persons whose illness met the WHO criteria for leptospirosis. Four people did not seek treatment at Khumuang Hosptial for their illness; their clinical information was not available. All 13 persons who sought treatment at Khumuang Hospital had a fever, 10 above 39°C. Other predominant clinical presentations included chills 84.6% (11/13), headache 76.9% (10/13), myalgia 84.6% (11/13), and calf pain 76.9% (10/13).

Descriptive Statistics and Univariate Logistic Regression

Univariate associations between exposures and leptospiral infection among the 104 persons sampled are presented in

Table 1. The median age of infected persons (35, range 15–65) was not significantly different from the median age of noninfected persons (40, range 15–63). Although the infection rate in women (32.7%) was lower than that of men (62.8%), the difference was not statistically significant (p=0.091).

In univariate analysis, the pond sites where people worked and places where lunch was eaten were not associated with infection. Infection was not significantly associated with the presence of hand wounds (p=0.092) or leg wounds (p=0.069). However, having more than two wounds anywhere on the

Table 1. Results of univariate analysis of potential risk factors for leptospirosis infection among persons participating in cleaning a pond: odds ratios (OR), 95% confidence intervals (CI), and chi-square p value

Risk Factor	Infected (n = 43) (%)	Noninfected (n = 61) (%)	OR (95%CI)	p value
Demographic				
Gender				
Male	27 (62.8)	28 (45.9)	1.99 (0.896 to 4.42)	0.091
Female	16 (37.2)	33 (54.1)		
Age in yrs (continuous)			0.970 (0.939 to 1.00)	
Individual				
Reported clinical illness	17 (39.5)	15 (24.6)	2.01 (0.862 to 4.67)	0.106
Location in the pond where work was preformed				
Site 1	5 (11.6)	10 (16.4)	0.67 (0.212 to 2.13)	0.498
Site 2	13 (30.2)	14 (23.0)	1.45 (0.602 to 3.52)	0.405
Site 3	15 (34.9)	30 (49.2)	0.55 (0.248 to 1.24)	0.149
Site 4	11 (25.6)	17 (27.9)	0.89 (0.367 to 2.16)	0.796
Site 5	14 (32.6)	14 (23.0)	1.62 (0.677 to 3.88)	0.279
Site 6	7 (16.3)	7 (11.5)	1.50 (0.485 to 4.64)	0.482
Site 7	10 (23.3)	11 (18.0)	1.38 (0.526 to 3.61)	0.514
Place where person ate lunch, pond rim vs. elsewhere	2 (4.7)	5 (8.2)	0.55 (0.101 to 2.96)	0.483
Clothing worn while working in the pond				
Shirt	443 (100)	60 (98.4)	*	0.986
Short sleeve shirt	10 (23.3)	7 (11.5)	2.34 (0.811 to 6.74)	0.116
Long sleeve shirt	33 (76.7)	53 (86.9)	0.500 (0.178 to 1.39)	0.183
Trousers	26 (60.5)	44 (72.1)	0.590 (0.258 to 1.35)	0.214
Long skirt	6 (14.0)	11 (18.0)	0.740 (0.250 to 2.17)	0.581
Trousers or long skirt vs. shorts	32 (74.4)	55 (90.2)	0.32 (0.107 to 0.940)	0.038
Any type of glove	1 (2.3)	2 (3.3)	0.700 (0.062 to 8.00)	0.776
Slippers	5 (11.6)	11 (18.0)	0.600 (0.192 to 1.87)	0.376
Tennis shoes or cut shoes	1 (2.3)	1 (1.6)	1.43 (0.087 to 23.5)	0.803
Boots	2 (4.7)	7 (11.5)	0.38 (0.074 to 1.91)	0.238
Boots filled with water	3 (7.0)	12 (19.7)	0.310 (0.081 to 1.16)	0.082
Any use of footwear	11 (25.6)	31 (50.8)	0.33 (0.142 to 0.778)	0.011
Wounds present while working in the pond				
Any hand wound	31 (72.1)	34 (55.7)	2.05 (0.889 to 4.73)	0.092
Hand wounds, 0–5 vs. 6 or more	12 (27.9)	11 (18.0)	1.76 (0.692 to 4.47)	0.235
Any trunk wound	2 (4.7)	2 (3.3)	1.44 (0.195 to 10.6)	0.721
Trunk wounds, 0–5 vs. 6 or more	1 (2.3)	1 (1.6)	1.43 (0.087 to 23.5)	0.803
Any leg wounds	10 (23.3)	6 (9.8)	2.78 (0.924 to 8.35)	0.069
Leg wounds, 0–5 vs. 6 or more	5 (11.6)	1 (1.6)	7.89 (0.888 to 70.2)	0.064
Any foot wound	3 (7.0)	9 (14.8)	0.43 (0.110 to 1.71)	0.232
Any wound	33 (76.7)	40 (65.6)	1.73 (0.716 to 4.19)	0.233
Total number of wounds dichotomized at the median, 2.0	21 (48.8)	13 (21.3)	3.52 (1.50, 8.30)	0.004

body was significantly associated with infection (p=0.004). Additionally, wearing trousers or long skirts was significantly protective against infection (p=0.038). Trousers or long skirts were worn by 48 (98%) of 49 women and 39 (70.9%) of 55 men (p=0.0002), and footwear was worn by 30 (61.2%) of 49 women and 12 (21.8%) of 55 men (p<0.0001).

Multivariate Logistic Regression

All variables associated with or protective against leptospirosis infection with a p value $\leq$0.1 by univariate analysis were included in the multivariable logistic regression model. No interaction was detected between any of the exposures. Age was retained in the model throughout the backwards elimination procedure to control for confounding. Sex did not confound any variables that remained in the model after backwards elimination and was removed from the final model. Although any type of footwear was protective in univariate, it was not independent in multivariable analysis. Multivariable analysis by using a backwards elimination procedure (p$\leq$0.05) while controlling for age indicated that having a total of more than two wounds anywhere on the body while working in the pond remained independently associated with infection ($OR_{adjusted}$=3.97, 95% CI 1.56 to 10.2), while wearing trousers or long skirts was protective ($OR_{adjusted}$=0.23, 95% CI 0.067 to 0.701) (Table 2). Multicolinearity was not detected among any of the variables in the multivariate model.

Table 2. Risk factors for leptospirosis infection by multivariable logistic regression controlling for age: odds ratios (OR), 95% confidence intervals (CI), and chi-square p values.

Variable	Adjusted OR	95% CI	p value
Age in yrs	0.980	(0.947 to 1.01)	0.247
Total number of wounds dichotomized at the median, 2.0	3.97	(1.56 to 10.1)	0.004
Long trousers or skirts vs. shorts	0.217	(0.067 to 0.701)	0.011

Discussion

Symptomatic *Leptospira* infection is often characterized as febrile illness accompanied by other symptoms including headache, conjunctival suffusion, muscle pains, and meningismus (26,27). Some persons may have clinically inapparent infection or symptoms too mild to be definitively diagnosed, especially in disease-endemic areas (7,28). The advantage of using the Lepto-Dipstick Test lies in its ability to detect serum IgM antibodies against *Leptospira*, showing recent infection. Studies have shown that antibodies against *Leptospira* develop 4–6 days after exposure and can be detected 3–6 months after illness; however, the length of persistence of the IgM antibodies is unknown (10,22–24). Based on IgM antibodies measured by the LEPTO-Dipstick test, our findings suggest that the proportion of asymptomatic infection for leptospirosis was 60.5% in this population. The asymptomatic infection rate reported here is consistent with other studies, which have shown asymptomatic infection rates up to 70.6% (19,27–30). Tangkanakul et al. reported a background asymptomatic infection rate of 8.4%–11% in a disease-endemic area of Thailand from August to December 1998 (31). This background rate is much lower than the asymptomatic infection rate that we found in our population, which may indicate that the pond may be the source of infection rather than some other reservoir. However, misclassification of persons as seronegative or seropositive may be a potential bias. Persons with asymptomatic infection are unlikely to be important in the transmission of leptospirosis, since person-to-person transmission is known to be rare in symptomatic patients (9,32). The full importance of subclinical or asymptomatic infection is not well understood, and efforts to determine its significance have been limited. Future studies in disease-endemic areas are needed to determine if asymptomatic infections may play a role in population immunity (herd immunity) against leptospirosis.

We found that the presence of more than two wounds remained independently associated with infection, while wearing trousers or long skirts was associated with protection against *Leptospira* infection in our multivariable model. This finding suggests that more than two wounds and the use of trousers or long skirts were the strongest independent predictors and protective factors for infection. The protective effect of the use of trousers or long skirts may be essential for intervention planning in Thailand. While previous studies have suggested the importance of broken skin in infection with leptospirosis, this study is the first that we are aware of to quantify the effect of skin wounds and suggest that risk may increase with increasing number of breaks in the skin (11,12). The location of a lesion was not significantly associated with infection in multivariable analysis, and data regarding severity of the skin wound were not collected. Broken skin probably facilitates the entry of *Leptospira* directly into the bloodstream and increases the number of bacteria that enter the host in a given exposure period.

These findings suggest that further education efforts are needed to encourage the practice of wearing protective clothing while working in areas of Thailand with endemic leptospirosis, and may have an application in the control of the nationwide epidemic. The significant association of more than two wounds with infection suggests that efforts are needed to reduce exposure to contaminated water when persons have open wounds are present. Our findings suggest that protective clothing and avoiding exposure to standing flood water by persons with open skin wounds may decrease the risk of leptospirosis in these settings.

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Outpatient Antibiotic Use and Prevalence of Antibiotic-Resistant Pneumococci in France and Germany: A Sociocultural Perspective

Stephan Harbarth,* Werner Albrich,† and Christian Brun-Buisson‡

The prevalence of penicillin-nonsusceptible pneumococci is sharply divided between France (43%) and Germany (7%). These differences may be explained on different levels: antibiotic-prescribing practices for respiratory tract infections; patient-demand factors and health-belief differences; social determinants, including differing child-care practices; and differences in regulatory practices. Understanding these determinants is crucial for the success of possible interventions. Finally, we emphasize the overarching importance of a sociocultural approach to preventing antibiotic resistance in the community.

The epidemiology of antibiotic-resistant *Streptococcus pneumoniae* varies tremendously between different countries and continents (1). In Europe, high rates of penicillin-resistant pneumococci have been recorded in France and Spain, whereas countries like Germany and Switzerland are only marginally affected (2,3). The reasons for the uneven geographic distribution of antibiotic-resistant pneumococci are not fully understood.

In this article, we focus on a comparison of pneumococcal resistance rates between Germany and France, two neighboring European countries with well-developed health-care systems accessible for virtually the entire population. Moreover, living standards, expenditures on health, and key survival statistics (infant deaths, life expectancy) are roughly equivalent, which allowed us to assume that at least in terms of general health indicators both countries could be judged to be comparable (4). We reviewed recent epidemiologic data about antibiotic resistance in clinically relevant pneumococcal isolates of patients in Germany and France and explored different hypotheses to explain the observed differences between the two countries. The main questions addressed are: 1) Do important differences exist in antibiotic-prescribing practices in the outpatient setting? 2) Do the factors influencing decisions on antibiotic use differ? 3) Are these differences related to sociocultural and other macro-level determinants? In particular, we sought to offer potential methods for future international comparisons designed to aid in developing effective strategies for decreasing the spread of antibiotic-resistant microorganisms in the community.

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Methods

A computer-based literature review was undertaken with the MEDLINE database from 1980 to the present. While references were sought by using specific subject headings related to differences in the prevalence of antibiotic-resistant pneumococci between Germany and France and reasons for the observed disparity (e.g., antibiotic use and prescribing), the paucity of relevant retrievals prompted us to repeat the search by using keywords specific for each of the questions asked. This extended search included articles about differences in economic and sociocultural determinants (e.g., perception of illness, societal background of pharmaceutical consumption). Additional references were identified from the references cited in these reports and personal files. Papers in English, German, and French were reviewed. Antibiotic use on a national level was expressed as defined daily doses (DDD) of different antibiotic agents per 1,000 inhabitants per day, one DDD being the standard daily dose of an antibiotic agent for 1 day's treatment (5).

Epidemiology of Resistant Pneumococci

Among all clinical isolates of *S. pneumoniae* collected from patients of all ages throughout Europe in 1998, 93% (n=168) were susceptible to penicillin (MIC $\leq$0.06 mg/L) in Germany, whereas only 47% of French isolates (n=167) remained fully penicillin-susceptible (6). In the same multinational study, 4% and 47% of pneumococcal isolates were erythromycin-resistant (MIC >1 mg/L) in Germany and France, respectively. A national surveillance study about the prevalence of penicillin-resistant *S. pneumoniae* recovered from patients with respiratory tract infections in Germany from 1998 to 1999 showed that of 961 isolates, 93% were fully susceptible to penicillin G and 6% had intermediate susceptibility (7). Three strains expressed high-level resistance to pen-

icillin (MIC >2 mg/L) in that study. In contrast, several recent reports confirm the high prevalence of antibiotic-resistant pneumococci in France (2,3,8–10). For instance, a national surveillance study conducted in France in 1999 demonstrated that the prevalence of penicillin-nonsusceptible (MIC >0.12 mg/L) and erythromycin-resistant pneumococcal isolates (n=14,178) were 43% and 51%, respectively (10). We found that the prevalence of antibiotic-resistant pneumococci is sharply divided between France and Germany. Figure 1 (A and B) summarizes currently available aggregate data on the prevalence of penicillin- and erythromycin-resistant pneumococci in clinical isolates from both countries (3,6,7,9–11).

Explanatory Dimensions

To explain the differences in pneumococcal resistance rates, we identified several dimensions that influence decisions on antibiotic use. These dimensions are derived from the concept that outpatient antibiotic use not only depends on clinical and microbiologic considerations and the frequency of respiratory tract infections but is also related to sociocultural and economic factors (12–14). More precisely, the first dimension of our proposed framework (Figure 2) concerns the prescribers of antibiotics, physicians, who may differ in their use, dosing, and choice of antibiotic agents. The second dimension concerns patient demand and health-belief differences. A third group of determinants of antibiotic consumption is linked to macro-level factors influencing the prescription of antibiotics, such as sociocultural factors (e.g., child-care practices) and regulatory health-care policies. This article has been structured along these explanatory dimensions. Finally, we discuss competing explanations and implications of the presented data.

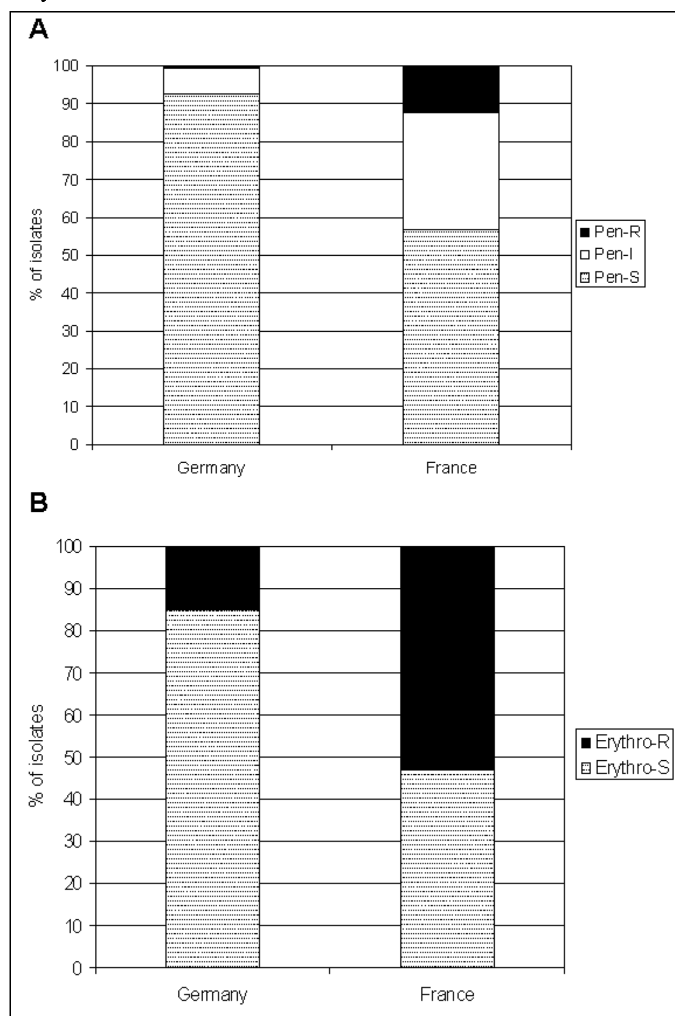

Figure 1. Summary of published aggregate data on the prevalence of pneumococci with intermediate (MIC >0.12 mg/L) and high-level (MIC >2.0 mg/L) resistance (A), and the prevalence of erythromycin-resistant pneumococci (B), France and Germany (3,6,7,9–11).

Volume of Outpatient Antibiotic Use

The association between community use of antibiotics and antibiotic-resistant pneumococci has been amply demonstrated (15–17). This relationship raises the question of whether differences in the volume and pattern of outpatient antibiotic use exist between Germany and France.

Analyses of national sales data from Germany and France are summarized in Figure 3 (18,19). These data show that, from 1985 to 1997, retail sales of oral antibiotics in France were almost three times higher than sales in Germany. For instance, in 1997, France used 36.5 DDD/1,000 population/day versus 13.6 DDD/1,000 population/day in Germany (Figure 3) (19). In addition, Germany had a higher relative use of narrow-spectrum penicillins, cotrimoxazole, and tetracyclines and a much lower use of broad-spectrum penicillins, cephalosporins, and fluoroquinolones, compared to France (2,19). Overall, among 18 industrialized countries, Germany had the third lowest and France had the highest antibiotic utilization rate in the outpatient setting throughout the 1990s (18,19).

Antibiotic-Prescribing Practices for Respiratory Tract Infections

Antibiotic-prescribing practices for respiratory tract infections vary tremendously between France and Germany. Antibiotic prescription rates in France and Germany for common cold and tonsillopharyngitis were 48.7 and 94.6 versus 7.7 and 69.6 per 100 outpatient consultations, respectively (Table) (12). A French survey showed that, during a 3-month period in 1991, 25% of the French population was treated with an antibiotic, compared with 17% in 1980 (22). In particular, the frequency of respiratory tract infections with a presumed viral cause that were diagnosed and treated with antibiotics increased by 86% for adults and by 115% for children in the 11-year period (22).

A pan-European survey showed marked differences in the rate of nonprescription of antibiotics at the first consultation for respiratory tract infections (Table) (20). In Germany, the absence of antibiotic prescription reached 41%, even in a case of a suspected pneumonia (21). This lower rate of prescriptions can be explained by a higher recourse to diagnostic investigations and a watchful waiting approach in Germany (21). In fact, in 43% of all suspected cases of respiratory tract infection in Germany, diagnostic tests were performed, compared to 21% in

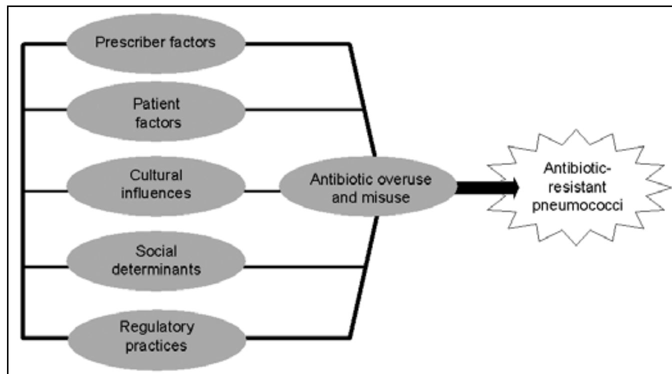

Figure 2. Framework of nonmicrobiologic factors influencing outpatient antibiotic use and prevalence of pneumococcal resistance.

France (Table). Another recently published study (23) confirmed that lower respiratory tract infections seen by general practitioners in France led to few requests for supplementary investigations. Thus, to decrease diagnostic uncertainty and inappropriate prescribing for acute bronchitis and mild exacerbation of chronic bronchitis in France, more precise diagnostic criteria and cost-effective tests are needed.

A study by Guillemot et al. (24) also demonstrated that frequent low-level prescribing of penicillin selects for resistant strains of *S. pneumoniae* in the oropharyngeal flora. When finally prescribed, penicillin agents are usually given in higher doses to German patients than French patients. In a European study, 30% of German adult patients received >3g per day of amoxicillin for lower respiratory tract infections, whereas French patients received considerably lower antibiotic dosages (21). In another survey from France, a high percentage of antibiotic prescriptions were underdosed as compared to clinical recommendations, particularly in children (25). Moreover, some authorities have linked the high prevalence of penicillin-nonsusceptible pneumococci in France to widespread replacement of amino-penicillins by oral cephalosporins, many of which achieve a T > MIC (time for which non–protein-bound concentrations exceed the MIC) of <40% for *S. pneumoniae,* resulting in inadequate killing of bacteria (26). In contrast, the prescription of high-dose amino-penicillins in Germany may be an additional factor contributing to the lower prevalence of penicillin-resistant pneumococci in that country (27).

The Cultural Perspective

Cultural factors determine which signs and symptoms are perceived as abnormal and thus require medical care and pharmaceutical treatment. Illness perception influences help-seeking behavior and clinical outcome (28). In particular, cultural views of infectious conditions that require antibiotic treatment differ between countries (14).

Many French people seeking medical care because of cough and sputum production request to be treated by antibiotics; by contrast, most Germans consider such treatment as unnecessary overmedication (Table) (12,29). In Germany, many patients accept individualized, complementary medicine and its most refined form, homeopathy, as an equivalent approach for the treatment of respiratory diseases, since great attention is given to improving the body's natural defense (30,31). A survey commissioned by the European Union among 1,577 opinion leaders in the health-care sector showed that alternative medicines such as homeopathy were supported by 42% of survey participants in Germany versus 23% in France (32). A recently published survey among 2,111 Germans >16 years of age showed that 83% had some sympathy for complementary medicine, whereas 40% disliked antibiotics because they could undermine natural immunity (33). Another opinion poll among 2,647 Germans indicated that the prevalence of using alternative medicine in Germany was the highest among all industrialized countries: 65% in 1996; in 1970, the corresponding figure was 52% (34). Most participants (84%) seemed motivated to use alternative methods largely because of strong misgivings about the potential adverse effects of pharmacotherapy (34).

Based on these health-belief factors, most German physicians follow a less aggressive, watchful-waiting approach in the case of non–life-threatening infections. In particular, German physicians agree that antibiotics are not first-line drugs for the treatment of uncomplicated respiratory tract infections. Indicative of the general attitude is this statement by a German general practitioner: "We never give antibiotics for a common cold ….On the first visit we would only give aspirin. After five days we would do a blood sedimentation and listen to the lungs. Then we might give antibiotics" (35).

In France, physicians have repeatedly reported that unrealistic patient expectations, patient pressure to prescribe antibiotics, and insufficient time to educate patients about the inefficacy of antibiotics for upper respiratory tract infections are the major reasons why antibiotics are prescribed for these self-limiting diseases (12,29,36,37). In a Pan-European survey (36), the demand index for antibiotics among patients in France was 2.2, surpassed only by Turkey (2.4). In that survey, France was the only European country where >50% of the interviewees definitely expected an antibiotic for the treatment of "flu." Most notably, 82% of French mothers expected antibiotics for their child's earache (36). In another recently published survey, French parents agreed more strongly than physicians that "all ear infections should be treated with antibiotics" (38).

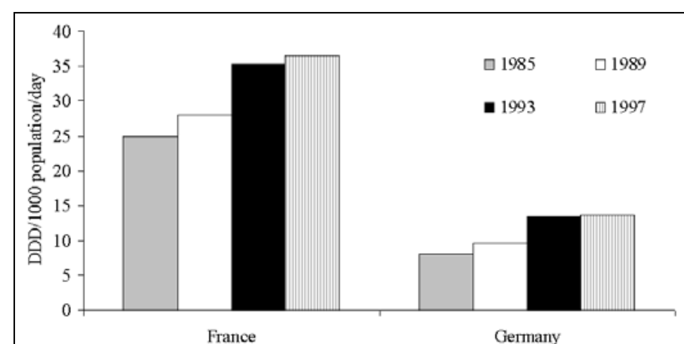

Figure 3. Outpatient antibiotic utilization (18,19), France and Germany, 1985–1997. DDD, daily defined doses.

Table. Comparison of patterns of antibiotic prescribing and diagnostic tests for respiratory tract infections, France and Germany[a]

Variables	France	Germany
Average no. office visits for acute tonsillopharyngitis per 1,000 population	136	51
Average no. antibiotic prescriptions per 100 office visits for acute tonsillopharyngitis	94.6	69.6
Average no. office visits for common cold per 1,000 population	253	19
Average no. antibiotic prescriptions per 100 office visits for common cold	48.7	7.7
% of patients not receiving antibiotics at first office visit for		
Suspected community-acquired pneumonia	8	23
Acute bronchitis	7	31
Exacerbation of chronic bronchitis	5	26
Viral lower respiratory tract infection	20	41
% of cases of suspected lower respiratory tract infection with diagnostic tests performed		
Chest radiograph	18	27
Peripheral blood leukocyte count	14	27
Microbiologic sputum examination	3	12

[a]Sources: (12,20,21).

However, French physicians may overestimate the extent to which patient satisfaction depends only on receiving an antibiotic prescription; therefore practitioners should be convinced that the primary determinant of patient satisfaction is not prescribing antibiotics but rather, effective communication about the patient's illness (39). For instance, in a recently published study from Nottingham, United Kingdom, antibiotic use for acute bronchitis was reduced by 25% in those patients who received information and reassurance about the benign nature of their disease (40).

The Social Perspective

Social factors also influence antibiotic use and resistance rates in France and Germany. This influence can be best illustrated by otitis media, the leading reason for excessive antimicrobial use in French children (20). Attendance at a child-care center outside the home correlates strongly with an increased risk of otitis media and acquisition of drug-resistant pneumococci (16,41). Therefore, the great differences in the availability and usage of nonparental day-care facilities between France and Germany are not unexpected.

In France, a long tradition of early childhood education exists in the public sector. Known as "écoles maternelles," nearly 100% of 3- to 5-year-olds attend these publicly funded pre-schools; about 35% of 2-year-olds also attend (42). In contrast, <10% of German infants in this age group were in the care of an external child-care provider (43). In 1998, 340 nursery places per 100,000 population were available in France, compared to 200 places per 100,000 population in Germany (43). Thus, many more French infants are in the care of an external child-care provider. If they enter child-care, German children enter it later than French children; this practice delays the peak incidence and the cumulative burden of otitis media and associated antibiotic use (44).

Because of the transmission of antibiotic-resistant pneumococci among infants in nurseries in France, a panel of national experts recommended encouraging alternatives that could delay placement in day-care centers until children are 18 months old (45). However, this recommendation seems difficult to follow, since attendance at a child-care center outside the home is a necessity for many families. In fact, France has a high proportion of women employed outside the home: in 1990, 72% of the women ages 25 to 54 years were employed in France compared to 60% in Germany (46). Moreover, <40% of single mothers in Germany are employed, compared to 82% in France (47). Since out-of-home child-care practices are unlikely to change and the proportion of two-career families will likely not decrease in France, promoting smaller child-care size, grouping children in small sub-units, and providing pneumococcal vaccinations could possibly reduce the risk of pneumococcal cross-infection (48–50).

By contrast with Germany, another important risk factor for otitis media and pneumococcal infection in infants (50) is highly prevalent in France: the absence of breast-feeding beyond the first weeks of life. Breast-feeding practices vary considerably throughout Europe (51). A national survey conducted in 1995 among 12,179 babies at French maternity hospitals showed that France had the lowest level among Western countries for which national data on breast-feeding were available: only 52% of newborns were breastfed at hospital discharge, including 10% of babies partially breastfed (52). Efforts to encourage breast-feeding are needed in France to promote infant health and decrease susceptibility to respiratory tract infections.

Differences in Regulatory Practices

Antibiotic prescriptions are affected through reimbursement policies and the structure of the pharmaceutical market.

The average level of retail prices for pharmaceutical products is very low in France. For example, if the price in France is 100, the level would be 162 in the United Kingdom and 175 in Germany (53). Another study (54) demonstrated that Laspeyres (U.S. quantity-weighted) indexes for prices per drug dose show large differences compared to the reference country (USA): Germany, +24.7%; Canada, +2.1%; Japan, –12%; Italy, –13%; United Kingdom, –17%; and France, –32%. Because of this low level of pharmaceutical pricing, France not only is ranked first in the consumption per capita of outpatient antibiotics but also had the 3rd highest consumption of pharmaceutical products per capita among all countries in the Organization for Economic Cooperation and Development in 1997 (55). The overall per-capita expenditures on pharmaceuticals in 1997, adjusted for cost-of-living differences, were $352 in France versus $294 in Germany (56).

Historically, the French drug economy has largely been regulated by product price control and has been structured as a low-price, high-quantity system, whereas Germany has tended more towards a high-price, low-quantity system (53,57). The different systems of price regulation are responsible to some extent for three important features that influence antibiotic prescribing patterns. First, generic medicines have played only a minor role (<5%) in the French pharmaceutical market (58,59), but they account for 39% of all prescribed medicines in volume and 38% in value in Germany (59). This feature contributes to the observed trend in France of using newer antibiotics; in Germany, by contrast, narrow-spectrum, generic agents are more commonly used (19). A second factor is that until recently, French pharmacies were better remunerated if they dispensed large volumes of relatively expensive drugs such as oral broad-spectrum cephalosporins (59). By contrast, pharmacy remuneration in Germany is calculated by applying regressive percentages to different price bands: the lower the price, the higher the pharmacist's share (60). Finally, the French pricing system has induced companies to develop aggressive promotional efforts and marketing campaigns to curb sales and compensate for low prices (53). Consequently, we speculate that French and German general practitioners are exposed to very different marketing information on antibiotics (12,29). However, representative data for both countries are not publicly available on that issue.

Most importantly, health authorities in Germany have more regulatory power by allocating collective expenditure caps and, therefore, have a broader impact on drug use than that exerted by similar agencies in France. In 1993, the introduction of capped physician budgets and a system of reference pricing in Germany led to a switch in prescribing preferences and an incentive for German physicians to avoid expensive products priced above the reference price, such as oral broad-spectrum cephalosporins (54). Consequently, from 1994 to 1997, the volume of antibiotics prescribed decreased temporarily from 334 million to 305 million DDDs (57).

In France, the introduction of national prescription guidelines (Références Médicales Opposables) for upper respiratory tract infections in 1994 did not decrease the overall volume of outpatient antibiotic use and had only a modest economic impact. However, prescription patterns have changed in line with those guidelines and led to a decrease in the use of fluoroquinolones and oral cephalosporins and to a substantial increase in macrolide use for acute bronchitis and pharyngitis (61).

Possible Alternative Explanations

Several alternative explanations for the observed differences in antibiotic resistance rates can be made. First, variation in antibiotic use may be caused by differences in the frequency of respiratory tract infections. However, the rate of antibiotic consumption in France implies a rate of bacterial respiratory illness that is at least 5 times higher than the reported rates in the literature (20); therefore, the high antibiotic usage cannot be justified by known rates of the principal bacterial infections of the respiratory tract encountered in the community. As stated by Guillemot et al. (22), the observed increase in respiratory tract infections with a presumed viral cause cannot be explained by demographic evolution, age distribution, or by the occurrence of large epidemics in France. Second, clonal differences may be responsible for the observed differences. However, we did not identify any study suggesting that the circulating strains and serotypes of antibiotic-resistant pneumococci in France are intrinsically more virulent or transmissible compared to strains circulating in Germany (62,63). Third, another possible reason for the epidemiologic gap between both countries might be differences in diagnostic practice. For instance, two recent studies suggest underdiagnosis of invasive pneumococcal disease in Germany (64,65). However, we have no reason to believe that France has a significantly higher detection and identification rate of pneumococcal infection, when considering the previously mentioned diagnostic practices in France (22,23). Finally, although obtaining comparable data about the severity of illness of outpatients in France and Germany is difficult, no evidence shows that the French health-care system is more likely to treat patients who are more severely ill or who have a higher likelihood of severe infection (66). Nevertheless, the heterogeneity of patient populations and their varying susceptibility to infection should be better described in future studies about international differences in antibiotic use and resistance rates.

Conclusions

This report represents a unique attempt to combine different data sources to give a more complete picture of sociocultural and economic forces influencing the ecology of antibiotic use and pneumococcal resistance in two large European countries. The published literature regarding the prevalence of antibiotic-resistant pneumococci provides convincing evidence that France and Germany have sharply different rate. The reasons for the observed resistance gap are multifactorial and include substantial differences in physicians' and patients' attitudes towards antibiotics; sociocultural and economic factors; and disparities in regulatory practices. Studies are

remarkably consistent in documenting the high frequency with which antibiotics are used in France for upper respiratory tract infections without appropriate microbiologic rationale. Unfortunately, despite the widespread publication of recommendations over the last decade and some modest modifications in the pattern of antibiotic utilization (61), the willingness of French general practitioners to change their antibiotic-prescribing habits has been at best grudging and at worst nonexistent (29,67). As shown in a recently published survey from the United States (68), little evidence suggests that national guidelines alone, particularly when they emphasize societal concerns, have much impact on individual antibiotic-prescribing behavior. Therefore, much more attention needs to be focused on patient expectations and perceptions of illness and the constraints of medical practice (39). Major improvements are needed in communicating to individual patients (69) and in informing the general public about the risks of inappropriate antibiotic use (13,70). In November 2001, the former French Minister of Health, Dr. Bernard Kouchner, took an important step in this direction by allocating 30 million euros for public awareness campaigns about antibiotic misuse and resistance (71). In this respect, France may also learn from the experience of countries like the United States, Canada, Belgium, and Sweden, which all managed to reduce excessive antibiotic use on a national level (19,72–74). In particular, a number of Scandinavian studies have suggested that national antibiotic policies together with public information campaigns and changes in reimbursement policies can be effective (55,75).

In Germany, low prevalence of pneumococcal resistance coincides with less antibiotic consumption, selection of narrow-spectrum antibiotics, higher dosing of amino-penicillins, and possibly, better treatment compliance (2). Thus, learning from Germany's experience with regard to the low prevalence of penicillin-resistant pneumococci may have some value for other countries. However, German health authorities should be careful in regard to the increasing spread of antibiotic-resistant pneumococci (11,76). A rational approach to the control of antibiotic-resistant pneumococci and the surveillance of antibiotic use in the outpatient setting are urgently warranted in Germany to preserve the still favorable situation.

An interesting question remains about whether differences in national antibiotic-prescribing patterns affect the rates of illness and death from complications of respiratory tract infections. A recent study (77) showed, for instance, that the Netherlands, a country with low antibiotic prescription rates for acute otitis media, had an incidence rate of acute mastoiditis of 3.8/100,000 person-years, whereas in countries with very high prescription rates, incidence rates were considerably lower, ranging from 1.2 to 2.0/100,000 person-years. A conservative approach and withholding of antibiotics in the treatment of acute otitis media may also have increased the occurrence of acute mastoiditis in Germany (78). A recently published article (79) indirectly suggests that the low rates of *Haemophilus influenzae* type B meningitis in some countries, particularly in Asia, may be due at least partially to extensive antibiotic use. However, no recently published, representative surveillance data exist about illness and death from complications of common respiratory tract infectious in France or Germany. Future international studies about the use of outpatient antibiotics (80) should include cross-country surveillance data for serious infectious complications such as mastoiditis, acute rheumatic fever, meningitis, or suppurative complications of pharyngitis (81).

Finally, we argue that effects exerted at the macro-level by the cultural and socioeconomic environment contribute substantially to the observed differences in prescribing practices and related antibiotic resistance rates. Consequently, failure to understand the sociocultural and economic perspectives of antibiotic consumption and resistance will lead to inadequate conclusions about the chances of success for possible interventions. More research to inform decision-makers on the determinants of the variation in antibiotic use and resistance patterns is urgently needed.

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Identifying Reservoirs of Infection: A Conceptual and Practical Challenge

Daniel T. Haydon,* Sarah Cleaveland,*Louise H. Taylor,* and M. Karen Laurenson*

Many infectious agents, especially those that cause emerging diseases, infect more than one host species. Managing reservoirs of multihost pathogens often plays a crucial role in effective disease control. However, reservoirs remain variously and loosely defined. We propose that reservoirs can only be understood with reference to defined target populations. Therefore, we define a reservoir as one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population. Existence of a reservoir is confirmed when infection within the target population cannot be sustained after all transmission between target and nontarget populations has been eliminated. When disease can be controlled solely by interventions within target populations, little knowledge of potentially complex reservoir infection dynamics is necessary for effective control. We discuss the practical value of different approaches that may be used to identify reservoirs in the field.

Infectious agents that can infect more than one host species are ubiquitous. Indeed, 62% of all human pathogens are classified as zoonoses (1), and 77% of livestock pathogens and 91% of domestic carnivore pathogens infect multiple hosts. Fifty seven of the 70 animal diseases considered to be of greatest international importance infect multiple hosts (2). The ability of pathogens to infect a wide range of hosts has been demonstrated as a risk factor for disease emergence in both humans (1) and domestic animals (2). Virtually all recent outbreaks of disease in endangered wildlife have been caused by pathogens that can infect other, more abundant host species 3,4.

Pathogens that infect more than one host species are by definition likely to be encountered in several host populations, some of which may constitute infection reservoirs. Therefore, a key issue in the design of control measures for multihost pathogens is defining what is meant by reservoirs of infection and developing guidelines for their identification.

Although many emerging diseases of human, domestic animal, and wildlife populations are assumed to be maintained in reservoir hosts (4), these reservoirs are rarely identified. In recent years, several emerging infectious disease threats to human and animal health have been managed through large-scale measures directed at suspected reservoirs of infection. Sometimes action arises from a clearly perceived notion of where infection resides. For example, approximately 1 million pigs were slaughtered in Malaysia in 1999 to control Nipah virus (5); several million chickens were slaughtered in Hong Kong in 1998 and 2001 to prevent a projected pandemic of *Influenza A virus* (6); and several million cows were slaughtered in Britain to curtail the epidemic of bovine spongiform encephalopathy, and its possible transmission to humans (7).

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However, many situations exist in which the role of reservoirs is less clear; for example, the reservoirs that harbor emerging viruses such as Ebola and Marburg remain unknown. For *Mycobacterium bovis* in the United Kingdom, a complex reservoir system seems most likely, and identification of the most important source of infection for cattle remains highly controversial (8). Incomplete understanding of reservoirs has hampered control of many diseases in Africa, such as Ebola virus infection, Buruli ulcer, and rabies (9–13).

Many different and often contradictory definitions of reservoirs exist. Studies stress different characteristics of reservoirs, namely, that infections in reservoir hosts are always nonpathogenic; any natural host is a reservoir host; the reservoir must be a different species; reservoirs are economically unimportant hosts; or reservoirs may be primary or secondary hosts (14–18). Some definitions imply that a reservoir comprises only one species; other definitions suggest that an ecologic system may act as a reservoir (16,18). Confusing, conflicting, and often incomplete concepts of what constitutes a disease reservoir result. We propose a conceptual framework for defining and identifying reservoirs and discuss the practical value of different approaches that may be used to identify reservoirs in the field.

Proposed Framework

We propose the following approach, which can be applied to any disease system, for understanding the role of potentially relevant reservoirs in that system. Figure 1 illustrates how this framework might be applied to various systems.

Suggested Terminology

The target population is the population of concern or interest to us. All other potentially susceptible host populations that

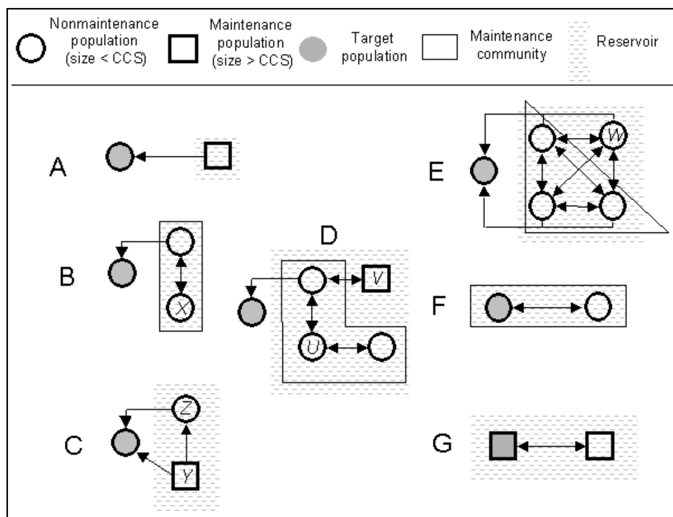

Figure 1. Examples of simple and more complex target-reservoir systems. In the simplest case, A, a maintenance population transmits a pathogen (indicated by arrows) to a target population that is smaller than the critical community size (CCS) and therefore classified as nonmaintenance. In B, the reservoir is composed of two connected nonmaintenance populations, only one of which is the source population, and neither of which could constitute a reservoir alone (typically akin to some vector-borne infections). Elimination of infection in population X will result in elimination of infection in the target. C depicts a situation in which Y is a maintenance population, but transmission can occur directly between Y and the target population or through another source population, Z. Although not essential to pathogen maintenance, Z is still part of the reservoir because it contributes to transmission of the pathogen to the target. In D, four nontarget populations must be included within the reservoir if its full dynamics are to be understood. Elimination of infection in U will not result in elimination of infection in the target, as V is an independent maintenance population. In E, all populations are sources. If W is not required to maintain the infection, then W falls outside the maintenance community but is still part of the reservoir because it is a source. F illustrates that the target population itself may constitute part of the reservoir and G that the target population can be a maintenance population.

are epidemiologically connected directly or indirectly to the target population are nontarget populations and could potentially constitute all or part of the reservoir. If we are interested in protecting humans (the target species) from cryptosporidiosis, for example, the wide range of domestic and wild animal species in the environment in which *Cryptosporidim parvum* occurs (19) is the nontarget population, and those species constitute potential reservoir hosts.

In epidemiologic theory, the critical community size is the minimum size of a closed population within which a pathogen can persist indefinitely (20). In smaller populations the number or density of infected hosts frequently falls to low levels, random extinction (fadeout) becomes inevitable, and the pathogen cannot persist. Populations smaller than the critical community size, or those rendered effectively smaller than that critical size through control measures, we term nonmaintenance populations. Pathogens will persist in populations larger than the critical community size, and these populations we term maintenance populations. In complex systems, pathogen transmission between a number of nonmaintenance populations could constitute a maintenance community. Any population that transmits infection directly to the target population, we define as a source population. Source populations may themselves be maintenance populations or, alternatively, may constitute all or part of a transmission link from a maintenance population to the target population.

If a target population is smaller than the critical community size and thus cannot maintain a pathogen, completely isolating the target population from any transmission from outside (ring-fencing) will cause the pathogen to become extinct in the target population. A reservoir is present if the pathogen repeatedly appears in such a nonmaintenance target population. For example, completely preventing tick transmission of *Borrelia* spirochetes to humans from other species would result in Lyme disease's disappearance from humans; thus, a reservoir must exist. This procedure for identifying reservoirs will not apply to maintenance target populations. However, in practical terms reservoirs generally only become of concern when disease control within the target population reduces transmission within a target population to a very low level relative to transmission from nontarget to target populations. For example, *Foot-and-mouth disease virus* (FMDV) is maintained in unvaccinated cattle populations in many parts of Africa. The identification of wildlife reservoirs (e.g., buffalo) generally only becomes important once vaccinated cattle can no longer maintain infection at the population level, as is the case, for example, in parts of southern Africa (21).

We propose that a reservoir be defined as one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population. Populations in a reservoir may be the same or a different species as the target and may include vector species. As long as a reservoir constitutes a maintenance community and all populations within the maintenance community are directly or indirectly connected to each other, the size of the reservoir has no upper limit.

Previous Concepts of Reservoirs

Previous reservoir definitions often required that the relevant infectious agent be nonpathogenic to the reservoir host species (14,15). However, pathogenicity, per se, has little bearing on the persistence of infectious agents in populations. Excluding the possibility of a reservoir solely because the infectious agent was pathogenic to a nontarget host—as is the case with pathogens such as Nipah, Hendra, and rabies viruses and with bovine spongiform encephalopathy—would clearly be a mistake.

Cleaveland and Dye (12) proposed criteria to identify reservoir hosts but did not take into account multihost aspects of reservoirs. Swinton et al. (16) used the terms reservoir and satellite to describe the dynamics of *Phocine distemper virus* in the North Sea population of harbor seals (*Phoca vitulina*). Infection from a satellite population effectively induces persistence of infection in the reservoir population (17). Neither population constitutes a maintenance population, but infection

can be maintained in a coupled system (illustrated in Figure 1B). Both satellite and reservoir populations would be components of our reservoir.

In an insightful paper, Ashford recognized many of the problems in the simplistic use of the term reservoir and proposed a consistent definition of a reservoir as an "ecological system in which the infectious agent survives indefinitely" (18). This definition differs from ours in that it does not reference a target population and thus does not require that a reservoir be a source of infection for a target population. Ashford defined reservoir hosts as those essential to maintenance of the pathogen. We, however, argue that reservoirs may include nonessential hosts. Excluding nonessential hosts from a reservoir causes two problems. First, populations harboring infection may be nonessential to maintenance yet play a major role in transmitting the pathogen to the target population. For example, FMDV persists indefinitely in African buffalo herds; yet impala may constitute an important source of infection for the cattle target population (22) (e.g., population Z in Figure 1C). Second, as Ashford recognized, the definition of reservoir membership becomes ultimately intractable if each constituent population in the reservoir is considered nonmaintenance. Under these circumstances, a reservoir could be composed of subsets of nonmaintenance populations in a variety of ways (Figure 1E). Although a minimal definition of a reservoir is clear, a fully inclusive definition is much less so. In Figure 1D, population V is not an essential host; nonetheless, this population must be considered a component of the reservoir because, if infection is eliminated in some other parts of the reservoir, eradication would not be achieved. For the same reason, our concept of a reservoir differs from the notion of a critical species assemblage, which is defined as the minimum set of host communities in which a parasite can persist (16).

Control of Infection

Practical disease control requires answers to two questions: 1) Can an acceptable level of control be accomplished without consideration of a reservoir? 2) If not, what populations constitute the reservoir? Given a target-reservoir system, policies to manage infection may contain elements of three broadly different tactics: 1) target control: directing efforts within the target population with no reference to the reservoir (e.g., human vaccination against yellow fever [23]); 2) blocking tactics: directing control efforts at blocking transmission between source and target populations (e.g., game fences to control FMDV in cattle); and 3) reservoir control: controlling infection within the reservoir (e.g., culling programs, vaccination, or treatment of reservoirs). These three approaches require progressively increased levels of understanding of reservoir structure and function.

Target control has the important advantage of requiring no knowledge of potentially complex reservoir dynamics. A complete understanding of infection dynamics within the reservoir is also not necessary to implement blocking tactics, although identifying source populations in the reservoir is essential. The more precisely that source populations can be identified and the more quantitative data that are available on their relative contribution to transmission, the more efficient the allocation of resources is for disease control. Reservoir control tactics require a much more complete understanding of the structure and transmission processes that occur within the reservoir. For example, efforts directed at controlling infection in nonmaintenance components of a reservoir are unlikely to be effective if infection in the maintenance component of the reservoir remains uncontrolled.

The practical problem of identifying reservoirs of rabies for humans in Zimbabwe provides a useful illustration of some issues involved. After a rise in the incidence of jackal and dog rabies in the 1990s, debate has centered on whether jackals (*Canis adustus*) are reservoirs of this disease, an issue that has important implications for formulating national rabies-control programs (10,11). In Zimbabwe, domestic dogs are a maintenance and source population of rabies for humans. However, jackals account for >25% of all confirmed rabies cases in animals and are also an important source of infection for humans (10,11). Jackals may be important components of the reservoir as a maintenance or nonmaintenance population (Figure 2). Because rabies can be maintained in dogs without jackals, jackals are not an essential constituent population of the reservoir. But can infection persist in jackals without dogs (Figure 2B)? Jackals may constitute part of a maintenance community in conjunction with an assemblage of other wild carnivores (Figure 2A). The question is important because if dogs are the only maintenance population in the reservoir, effective vaccination campaigns targeted at dogs should successfully eliminate human rabies from Zimbabwe. If, however, jackals comprise all or part of a maintenance community independent of dogs, eliminating rabies will only be successful if jackal rabies were also controlled (10,11). The recent high incidence of jackal rabies in Zimbabwe might suggest that jackals are maintenance populations. A high incidence of disease alone is neither necessary nor sufficient evidence for this claim, particularly when wide fluctuations in disease incidence occur (as with jackal rabies). Mathematical models suggest that jackals are probably unable to support infection without frequent reintroductions from outside sources (24). However, Bingham et al. (11) argue that spatial patterns are critical and that jackal epidemics may be sustained independently within key geographic areas. The issue can be resolved unequivocally through implementation of a mass dog vaccination campaign, which would be a logical first phase of a national program. If jackal rabies persists in the absence of dog rabies, an effective program for rabies elimination will likely need to include oral vaccination of jackals.

Rabies also provides an example of the need to identify a target population when defining reservoirs. In the Serengeti Plain in Tanzania, a distinct strain of rabies appears to be maintained independently in spotted hyenas, without causing them any clinical disease, and with no evidence of spillover infection or disease occurring in any other species (within the

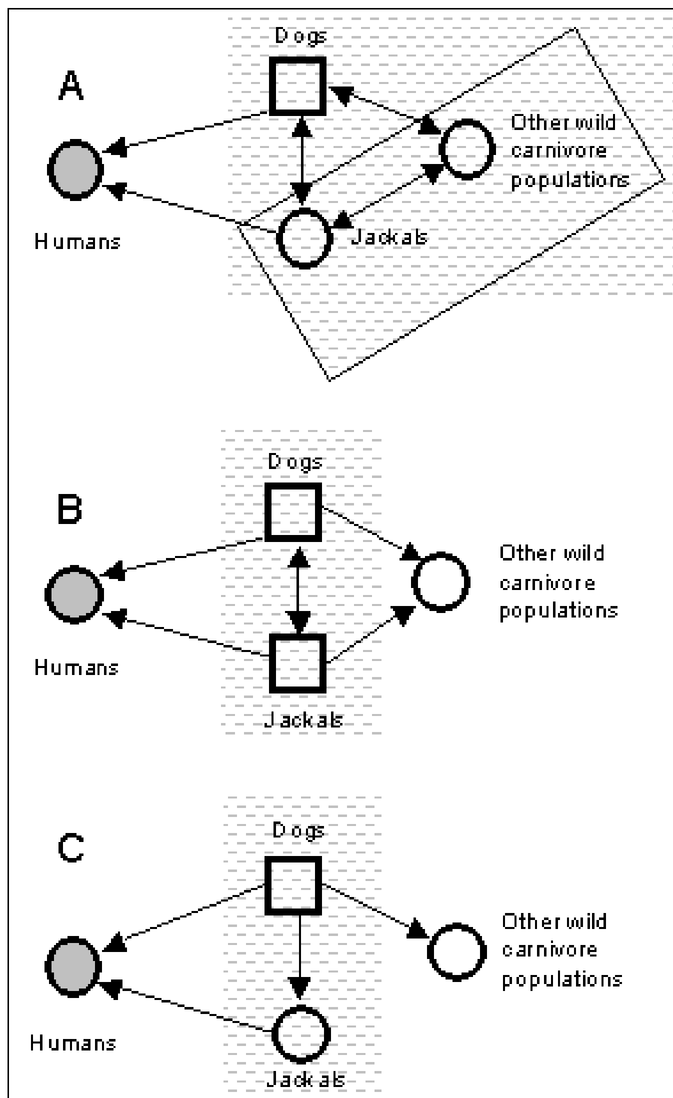

Figure 2. Potential complexity of rabies reservoirs in Zimbabwe. If jackals with (A) or without (B) other wild carnivore populations constitute a maintenance community independent of dogs, then vaccination of dogs alone will not result in rabies elimination in the target. If jackals do not constitute a maintenance community independent of dogs (C), then dog vaccination should clear rabies from the reservoir (symbols as in Figure 1).

limits of current knowledge) (25). By our definition, unless this strain is identified as the cause of disease in another species (i.e., a target population), hyenas in the Serengeti cannot be considered as a reservoir of rabies.

Practical Indicators To Identify Reservoirs

Newly emerging diseases usually originate from reservoirs of infection in other host species. When such diseases first appear, only rapid, accurate identification of the reservoir will enable appraisal of the full range of disease-control options. Ring-fencing is clearly impractical when no knowledge of the reservoir populations exists, but other steps can be taken to acquire progressively more detailed information about the reservoir structure.

Epidemiologic Evidence of Association

Accumulating epidemiologic evidence is often the best first step in identifying a reservoir. Initially, such analyses are often based on sparse data and are rarely published. Links between target and reservoir may be particularly elusive when transmission from reservoir to target is rare or sporadic, as, for example, occurs with Ebola virus or *Marburg virus* (26).

Quantitative data on risk factors for infection can be obtained through more formal epidemiologic research, such as case-control and cohort studies. For example, a case-control study of Borna disease in cats indicated that hunting mice was a risk factor and that rodents might be virus reservoirs (27). Case-control studies have identified badgers as risk factors for *M. bovis* infection in cattle in some parts of the United Kingdom (28). In other cases, putative reservoirs have been ruled out. For example, a risk factor analysis of *Helicobacter pylori* infection in young children showed that household pets were not incriminated (29). Although such associations may suggest a link between reservoir and target populations, further evidence is required to establish the identity of a reservoir.

Evidence of Natural Infection in Nontarget Populations

Identifying natural infection is a useful step towards determining natural hosts that may constitute potential reservoirs. Natural infection may be determined in two ways: by identifying previous infection through antibody detection or by identifying current infection through isolating the infectious agent or its genes from the host. The appropriate approach depends on the longevity of the infection in the host and the resources available. For example, very large sample sizes might be required to isolate a virus from a reservoir population; a serologic survey might be less expensive and more feasible. In a number of studies, demonstration of natural infection has been considered strong evidence that hosts are reservoirs, e.g., *Leishmania* in small mammals in Iran (30) and hantavirus in rodents in the Americas (31).

Seropositivity indicates that infection has occurred. However, not all natural hosts are reservoir hosts, and to include a nontarget population in a reservoir, evidence of transmission to the target population, direct or indirect, must exist. Furthermore, the level of seroprevalence does not provide information as to whether a nontarget population is a maintenance host. High seroprevalence at a single point in time may simply indicate an outbreak in the host population, rather than pathogen persistence (32). Low seroprevalence may arise when case-mortality rates are high in the reservoir (as in rabies infections), during an interepidemic trough, or when a pathogen persists at a stable but low prevalence, particularly when the duration of the infectious period is high (e.g., as in carrier animals). The critical issue is the persistence of infection in the reservoir, which can only be determined through longitudinal studies.

Similar guidelines apply to data based on demonstration of the pathogen within a host. For example, detection of *Trypanosoma brucei gambiense* in wild ruminants and primates in

West Africa has been taken as evidence of an animal reservoir for Gambian sleeping sickness (33). However, as animal-to-human transmission has never been demonstrated, wildlife remain classified as potential reservoir hosts, and disease control relies on treatment of people. In contrast, for Rhodesian sleeping sickness, isolation of *T. brucei rhodesiense* from a single bushbuck in the 1950s (34) led to the assumption that wildlife was the principal reservoir for human disease and resulted in widespread culling of wildlife for disease control. Only in 1966 were cattle identified as reservoir hosts (35). Current strategies focus on treating cattle with trypanocidal drugs (36).

Detecting a pathogen, particularly its transmission stage, in secretions or tissues provides supportive, but not unequivocal, evidence that transmission to the target population can occur. Even where experiments demonstrate that transmission is possible, it may not occur in nature for a variety of behavioral or social reasons, because the population is below critical community size or because of constraints of pathogen life history.

Genetic/Antigenic Characteristics

Genetic and antigenic characterization of pathogens isolated from different populations provides a more powerful tool for identifying key components of reservoirs. Antigenic and genetic variation of pathogens isolated from the target population within the range observed in the reservoir is consistent with reservoir-target transmission**.** This pattern can be demonstrated by applying phylogenetic methods to sequence, random amplified polymorphic DNA, or restriction fragment length polymorphism data, or by using serum cross-reactivity studies. Such methods have also been used to rule out important animal reservoirs of human disease in studies of *Ascaris* in Guatemala (37) and *Cryptosporidium* in Australia (38).

Intervention Studies

Complete ring-fencing of target populations is the ultimate step in identifying the existence and structure of reservoirs. In practice, however, ring-fencing has rarely been achieved and, as a result, even those reservoirs we consider to be most fully understood are not usually incontrovertibly proven. Despite this, once a potential reservoir is identified, intervention studies can permit incidental but powerful inferences about the dynamics of infection in target-reservoir systems. In many cases, disease-control programs can effectively act as intervention studies.

Control in a reservoir host population may be achieved by reducing host or vector density (e.g., culling possums to control tuberculosis in New Zealand [39], mosquito control for West Nile fever, or sandfly control for cutaneous leishmaniasis [40]). Alternatively, control measures may focus more directly on preventing transmission from the reservoir, e.g., separation of cattle and wildebeest to prevent transmission of malignant catarrhal fever in East Africa (41). The success of such interventions often provides reasonable confirmation of the original assumptions concerning transmission and maintenance of infection in the target-reservoir system.

Conclusions

We have a poorer understanding of the epidemiology of multihost pathogens than simpler single-host systems. This dearth of understanding is a particular problem with emerging diseases, since most emerging human, domestic animal, and wildlife diseases infect multiple hosts. Reservoirs must be defined with reference to particular target populations. Disappearance of the pathogen in the target population after ring-fencing provides categorical evidence of the existence of a reservoir and its possible identity. However, exhaustive identification of all constituent populations of a reservoir may be difficult. This identification need not be a management priority if disease control is directed at the target population or at blocking transmission between reservoir and target. For infection to be eliminated, however, disease-control measures must be directed at the reservoir. Thus, an understanding of reservoir infection dynamics is essential.

When the risks and costs of control are low, circumstantial evidence may be sufficient to justify implementing control measures. Specifically designed intervention studies have ultimately been required to determine whether a particular species is a maintenance host, a source of infection, or one that has been infected incidentally. Control measures are likely to be ineffective if they are directed at components of the reservoir that are neither maintenance hosts nor transmitters of the pathogen to the target population.

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PERSPECTIVE

Dengue Hemorrhagic Fever in Infants: Research Opportunities Ignored

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The age distribution of cases of dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) in infants under the age of 1 year are reported from Bangkok, Thailand, and for the first time for Ho Chi Minh City, Vietnam; Yangon, Myanmar; and Surabaya, Indonesia. The four dengue viruses were isolated from Thai infants, all of whom were having a primary dengue infection. Progress studying the immunologically distinct infant DHF/DSS has been limited; most contemporary research has centered on DHF/DSS accompanying secondary dengue infections. In designing research results obtained in studies on a congruent animal model, feline infectious peritonitis virus (FIPV) infections of kittens born to FIPV-immune queens should be considered. Research challenges presented by infant DHF/DSS are discussed.

Since World War II, the four dengue viruses (formal name: *Dengue virus* [DENV]) have progressively spread geographically throughout the tropics, resulting in a global pandemic with tens of millions of infections annually, including several hundred thousand hospitalizations for dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (1). The size and spread of the dengue pandemic, the unpredictability of epidemic occurrences, and the circulation of virulent and nonvirulent strains make DHF/DSS a model for an emerging infectious disease.

Ample evidence suggests that DHF/DSS accompanies secondary dengue infections in children older than 1 year (1–3). Less well-known are the epidemiologic and clinical studies that document an identical severe syndrome in infants during their first dengue infection (4,5). Ignoring these data, contemporary models of dengue immunopathogenesis focus on the sequential dengue viral infection phenomenon; such models suggest that severe disease results from amplified cytokine release caused by dengue infections occurring in the presence of T-cell memory (6). However, that model cannot explain DHF/DSS during a first dengue infection.

That dengue in infants is not often studied is understandable. Small subjects pose technical difficulties in obtaining samples required by research protocols, and human use protocols may be constraining. Yet infants represent 5% or more of all DHF/DSS patients (7). Uniquely, infants with DHF/DSS present an opportunity to obtain both the causative virus and the preinfection antibodies as research reagents in a hospital setting without recourse to a time-consuming and expensive prospective cohort study. The all-important preinfection antibodies can be collected from the mother, as her serum is a surrogate for cord blood (8).

Enhancement of infant infectious diseases by cord blood antibodies is not described for human infections other than dengue. However, such a phenomenon occurs naturally in infected kittens born to queens immune to feline infectious peritonitis virus (FIPV) (9–11). To refocus attention on the research opportunities afforded by this immunopathologic entity, we provide evidence that infants with DHF/DSS are regularly admitted to hospitals in four of the largest dengue-endemic countries. The age distribution of all these infant DHF/DSS patients is similar. Most of those studied serologically had had primary dengue infections. Because of FIPV's congruence to infant dengue, a short literature review is provided on that animal model.

Materials and Methods

Patients

Data on infants, ages <12 months, hospitalized with a clinical diagnosis of DHF were obtained from four hospitals: Children's Hospital No. 1, Ho Chi Minh City, Vietnam; the Queen Sirikit National Institute of Child Health, also referred to as Bangkok Children's Hospital, Bangkok, Thailand; Children's Hospital, Yangon, Myanmar; and the Department of Pediatrics, Dr. Soetomo Hospital, Surabaya, Indonesia. In this study, data for 4 consecutive years, either 1995–1998 or 1996–1999 were combined. Patients were under the routine care of one or more of the authors, each an experienced senior academic infectious diseases pediatrician. All diagnoses of DHF/DSS in infants conformed to World Health Organization case definitions. In Bangkok, serum samples from all infants and children hospitalized for DHF were sent for routine diagnostic study to

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the Virology Department, Armed Forces Research Institute of Medical Sciences (AFRIMS). For nearly 30 years, AFRIMS has provided such dengue diagnostic services to Bangkok Children's Hospital. Similar routine diagnostic tests were provided for infants and children admitted to Children's Hospital, Yangon, by the Virology Department, Department of Medical Research. Fiscal constraints limited the number of serologic tests performed. Individual data were disassociated from any identifiers and are presented here only in aggregate.

Virus Isolation

As described, DENV isolations were attempted from acute-phase plasma or serum samples from Thai children by inoculation into C6/36 cells or intrathoracically in mosquitoes (*Toxorrhynchites splendens)* (12).

Viral Identification

DENV was identified in C6/36 cells by an antigen-specific enzyme-linked immunosorbent assay (ELISA) with a panel of monoclonal antibodies against DENV (13).

Serology

Plasma or serum samples were tested for serologic evidence of acute DENV infection by immunoglobulin (Ig) M and IgG ELISA, hemagglutination inhibition (HAI) assays, or both (14). For single specimens, ≥5 days after onset of fever 40 U of IgM to DENV was considered evidence of a DENV infection. A DENV IgM-to-IgG ratio ≥1.8 defined a primary infection. A ratio <1.8 defined a secondary DENV infection. With serial specimens, twofold increase in IgG to DENV with an absolute value of ≥100 U indicated a secondary infection in the absence of IgM to DENV of ≥40 U.

In Bangkok, HAI antibody against DENV types 1–4 and *Japanese encephalitis virus* were measured in all sera (15). A fourfold increase was considered positive for acute flavivirus infection. The infection was diagnosed as primary if titers ≥1 week after onset of illness were ≤1:1,280 or as secondary if antibody titers were >1:1,280 (16).

Results

Infants are at high risk for DHF/DSS. Figure 1 provides data from the only published study to estimate age-specific dengue hospitalization rates for the Bangkok metropolitan area. In 1964, 17/1,000 seven-month-old infants, more than 1% of the population that age, were hospitalized for DHF/DSS (17). This modal rate was two times higher than the 1964 modal hospitalization rate for children (age 4 years, data not shown) in Bangkok during the same year (17). In our present study, infant DHF/DSS constituted 4.9%, 4.6%, 5.0%, and 4.9% of 4,872; 14,053; 8,938; and 2,057 Thai, Vietnamese, Myanmar, and Indonesian infants and children hospitalized with DHF in 1995–1998, respectively.

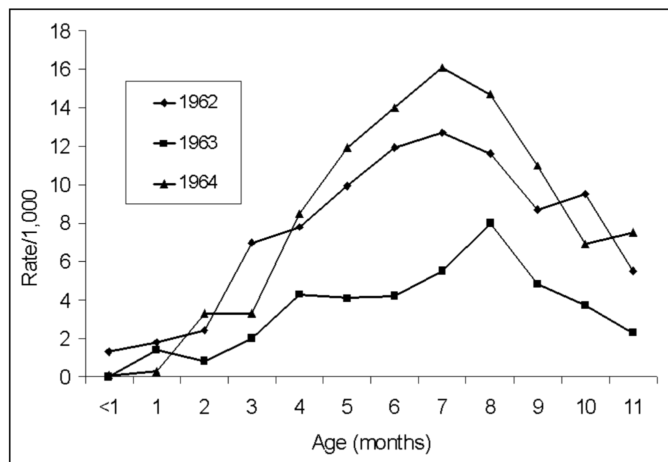

Figure 1. Age-specific hospitalization rates/1,000 infants with dengue hemorrhagic fever/dengue shock syndrome, Bangkok, Thailand, 1962–1964. Source: Halstead SB, et al. Am J Trop Med Hyg (17); cited with permission.

During a 4-year period, 237, 652, 449, and 101 infants with presumptive DHF/DSS were admitted to hospitals in Bangkok, Ho Chi Minh City, Yangon, and Surabaya. No significant differences in age distributions were observed year to year (data not shown). Data for 4 years were combined to smooth age distributions (Figure 2). Among infants hospitalized at Bangkok Children's Hospital, the distribution of World Health Organization grades 1, 2, 3, and 4 was 16.4, 56.2, 23.9, and 3.0%, respectively. Only 2 of 220 infants whose serum samples were tested had a secondary-type antibody response, 97.5% had primary infections. DENV was isolated from 114 (nearly 50%) of Thai infants; DENV types 1, 2, 3, 4 were recovered from 34, 23, 56, and 1 infant, respectively. Nineteen Myanmar infants were serologically confirmed as having a recent primary infection; seven, mostly infants ≥10 months of age, had secondary-type dengue HAI-antibody responses.

The distribution by age of DHF/DSS infants in all four countries presents a similar pattern: few cases were observed in infants younger than 3 months, and the largest numbers observed were in infants 6–8 months old. Later in the first

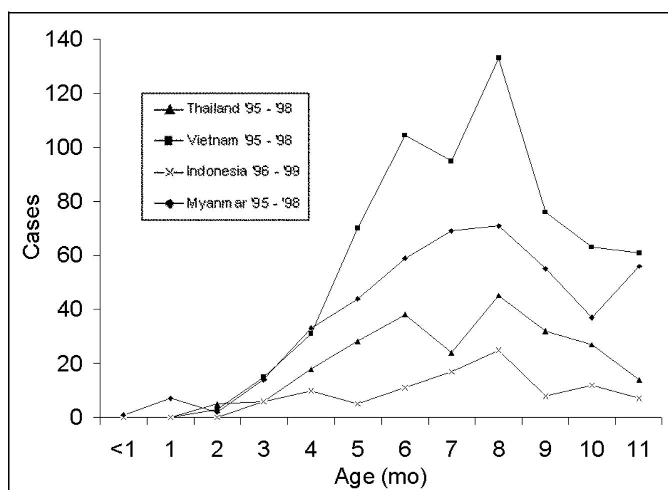

Figure 2. Month of age of infants hospitalized for dengue hemorrhagic fever/dengue shock syndrome at the Bangkok Children's Hospital, 1995–1998 (Thailand), Children's Hospital No.1, Ho Chi Minh City, 1995–1998 (Vietnam), Yangon Children's Hospital, 1995–1998 (Myanmar) and Dr. Soetomo Hospital, Surabaya, 1996–1999 (Indonesia). Data are combined for the period shown.

year, admissions declined nearly to baseline in Thailand and Indonesia. In Yangon, the decline in admissions reversed at age 10 months and increased. In Yangon, DHF hospitalizations continued to increase during the second year of life (Figure 3). In Ho Chi Minh City, cases in 11-month-olds declined, but not quite to the baseline.

Children hospitalized with DHF/DSS in Bangkok show the classical bimodal curve: relatively few cases in children 12–24 months of age and a modal age later in life, in this case at 8 years of age (Figure 4). By contrast, this bimodal distribution is not present in Yangon. DHF/DSS occurs commonly in 12- to 24-month-old children, and the modal age at admission is 4 years (Figure 3).

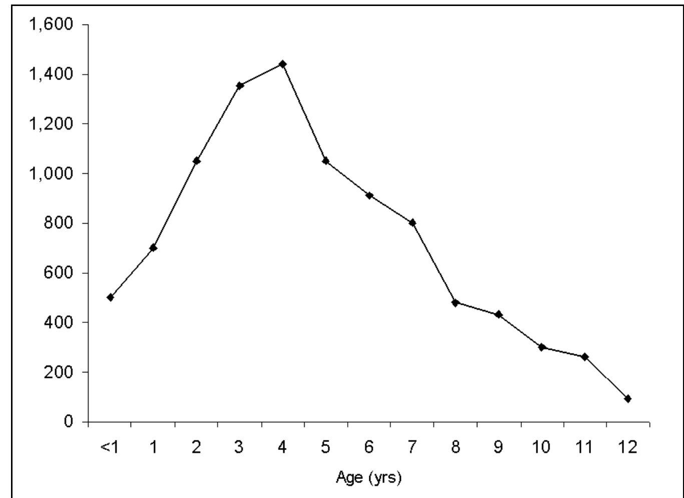

Figure 3. Year of age at hospitalization of children with dengue hemorrhagic fever/dengue shock syndrome, Yangon Children's Hospital, Yangon, Myanmar, 1995–1998, combined.

Discussion

The patterns of age distribution of infant DHF/DSS were similar in four large Southeast Asian countries highly endemic for all four DENV serotypes. Nearly all infants in the large Thai and most in the smaller Myanmar groups had primary DENV antibody responses. The characteristic and unique age-specific hospitalization curves are consistent with published observations that describe infant DHF/DSS occurrence during primary DENV infections. Primary and secondary DHF infections are reconciled in a long-standing explanatory hypothesis linking severe disease to actively or passively acquired antibodies (4,8,18). In our study, infants constituted approximately 5% of total DHF/DSS patients, lower than the nearly 10% reported from Bangkok Children's Hospital in the 1960s (7,17).

Differences were observed in the age distribution curves in Yangon and Ho Chi Minh City compared with Bangkok and Surabaya. In Yangon, the curve declined at 8 months but rose again at age 10 months, and no dip in cases occurred in 1-year-old children (Figures 2 and 3). In Ho Chi Minh City, infant cases declined at the end of the first year of life, but not to the baseline; by contrast, in Thailand and Indonesia by the end of the first year of life, the curve approached the baseline. These phenomena may be explained by differences in average annual rates of dengue infection. In Yangon, the modal age of hospitalization for DHF/DSS for children is 4 years (Figure 3), while in Bangkok it is 8 years (Figure 4). In Bangkok, DHF/DSS is rarely seen in 12- to 24-month-olds, signifying that second infections are usually delayed until after a child has lived through two dengue transmission periods (Figure 4). Among serologically studied infant DHF/DSS patients in Yangon, several ages 10 months and older had secondary dengue infections. These observations are consistent with high average annual rates of dengue infection in Yangon and lower infection rates in Bangkok. These relationships have been modeled mathematically (19).

Since infant DHF/DSS was first reported in 1970 (4), only a single research study has been undertaken on this group (8). This study included 13 Bangkok infants, all with primary DENV-2 infections, who were admitted to hospital at different ages in the first year of life. During hospitalization, mother's blood was taken and tested as a surrogate for cord blood at birth. An analysis of dengue-neutralizing antibodies showed that every mother in the study had had two or more previous DENV infections (8). All infants acquired DHF/DSS during the short window of time when maternal DENV-2 neutralizing antibodies had degraded to a titer of approximately 1:10. Maternal sera enhanced DENV-2 at high dilutions. These data provide a logical explanation for the observed age distribution of infant DHF/DSS. At birth, maternal antibodies protect infants from dengue infection. As IgG antibodies are catabolized, a period of risk to enhanced infection ensues, followed in turn by the loss of enhancing antibodies and a corresponding decline in risk for DHF/DSS (Figure 5).

Data from studies on infants as well as prospective cohort studies on children demonstrate that the waning or absence of heterotypic neutralizing antibodies permits enhanced infections to occur. Infection enhancement occurs at lower antibody concentrations than neutralization (8,20).

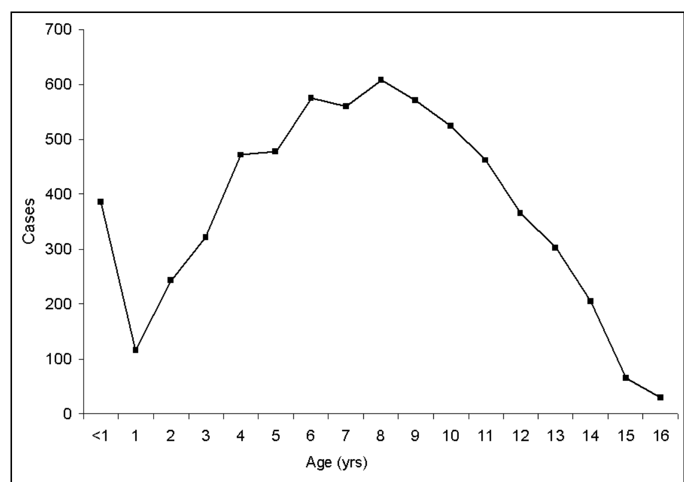

Figure 4. Year of age of children hospitalized for dengue hemorrhagic fever/dengue shock syndrome at Bangkok Children's Hospital, Bangkok, Thailand, 1990–1999, combined.

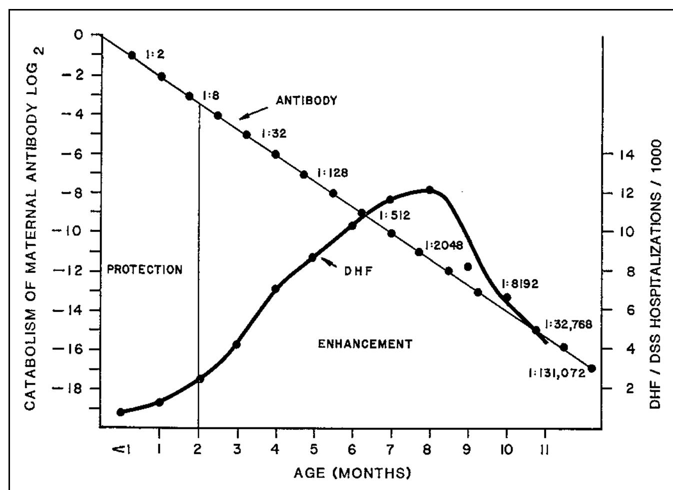

Figure 5. Relationship between the age distributions of infants hospitalized for dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) and the protective and infection-enhancing effects of maternal dengue antibodies. Shown are mean age specific hospitalization rate/1,000 for Bangkok and Thonburi, 1962–1964 (see Figure 1). At birth, antibodies are at protective concentrations. With the passage of time, maternal immunoglobulin G antibodies are catabolized to concentrations that result in antibody-dependent enhancement (ADE) of infections. By the end of the first year of life, ADE antibodies are catabolized to concentrations below the ADE threshold, and DHF/DSS cases disappear.

Immunopathogenesis mechanisms have been more extensively studied in a remarkably similar viral infection of cats, FIPV, a highly fatal coronavirus disease of domestic and exotic cats (9–11). Most cats naturally exposed as adults to FIPV develop antibody titers without showing clinical signs. Lesions in sick cats are believed to result from immunologically mediated responses (9,10,21). Kittens receiving apparently competent neutralizing antibodies to FIPV, transferred in colostrum from immune queens, develop a fatal disease a few days after infection with wild-type virus (22). Passive transfer of antibody by other routes produces the same result (22,23). This phenomenon is called the early death syndrome. FIPV in antibody-negative kittens occurs less reliably and is delayed for several weeks until animals develop their own antibody response to the virus. FIPV in kittens is characterized by thrombocytopenia and elevated ALT, AST, and serum bilirubin (22).

The coronaviruses, pathogens of mammals and birds, are a large family of enveloped RNA viruses with a nonsegmented, positive-stranded genome that is 27–32 kb in length (24). One of the most intriguing aspects of coronavirus replication is the occurrence of high-frequency homologous RNA recombination (25). Together with porcine *Transmissible gastroenteritis virus* (TGEV), *canine coronavirus*, and human coronavirus 229E (HCV), the feline coronaviruses form a separate cluster within the genus *Coronavirus,* including Feline enteric coronavirus, and FIPV (26). Coronavirus virions possess three structural proteins, a large spike glycoprotein (S), a small integral membrane glycoprotein (M), and a nucleocapsid protein (N) (24). These proteins are analogous to the envelope (E), M, and nucleocapsid (C) proteins of the flaviviruses. The feline coronaviruses can be divided into two serotypes, I and II, on the basis of cross-reactivity to *canine coronavirus* in virus neutralization assays (26). Type I viruses grow poorly in tissue cultures and show virtually no neutralization with anti–*canine coronavirus* sera (27). Type II viruses grow readily in vitro (28). Analysis of gene structure suggests that type II viruses are derived from recombination of type I feline coronavirus and *canine coronavirus* (26). The two serotypes circulate as two pathotypes, the avirulent enteric viruses and the virulent FIPV (26). High-frequency mutations may help coronaviruses escape neutralization and promote infection enhancement in Fc receptor–bearing cells.

Antibody-dependent enhancement of FIPV has been demonstrated in vitro in feline macrophages as well as in stable human and mouse macrophage cell lines (29). More cells are infected in the presence compared with the absence of antibody; the rates of viral entry and viral replication are similar under both conditions (30). Coronaviruses appear to enter mononuclear phagocytes by means of the plasma membrane without marked involvement of phagocytic or endosomal pathways (28). Some researchers have surmised that, as with DENV, when antibody-virus complexes attach to Fc-receptors, viruses are brought close to cell surfaces, where they enter the cells by normal mechanisms (31,32). Enhancement is mediated by clusters of epitopes on the S protein (33,34). Results with FIPV suggest that feline IgG2a antibodies mediate both neutralization and enhancement (33). Antibody-dependent enhancement in FIPV demonstrates a bell-shaped curve with increasing dilutions; maximal enhancement occurs at subneutralizing titers (34).

Antibody-dependent enhancement is believed to be the cause of vaccine failure after immunization with live (35,36) or recombinant (37) vaccines. Inoculation of cats with a recombinant vaccinia virus expressing the S protein FIPV 79-1146 sensitized cats and led to accelerated disease after FIPV challenge (37), while inoculation with recombinant vaccinia viruses expressing the M or N proteins did not (38). Immunization with vaccines made from other members of the feline coronavirus group, TGEV or *canine coronavirus,* also sensitizes cats to early death syndrome (39).

Flaviviridae do not appear to be subject to as high rates of homologous recombination as are the *Coronaviridae*; nonetheless, during evolutionary history four dengue serotypes have emerged. A phenomenon reminiscent of the feline coronaviruses is the evidence that DENV also circulate as two biotypes: DENV-2 American genotype does not cause DHF/DSS, while DENV-2 SE Asian genotype does (40). As with feline coronaviruses, the severity of disease with the two DENV biotypes may be regulated by cross-reactive antibodies (41). Focused research on viral-antibody interactions at the structural level might clarify early pathogenesis events in DHF/DSS. Infants may provide an accessible and inexpensive model to study mechanisms controlling the severity of dengue infections. Workers actively involved in developing dengue vaccines may benefit from lessons learned in the FIPV model.

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PERSPECTIVE

Role of the Domestic Chicken (*Gallus gallus*) in the Epidemiology of Urban Visceral Leishmaniasis in Brazil

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Zoonotic visceral leishmaniasis (ZVL) is a serious public health problem in several Brazilian cities. Although the proximity of chicken houses is often cited as a risk factor in studies of urban ZVL, the role chickens play in the epidemiology of the disease has not been defined. Chickens attract both male and female sand flies (*Lutzomyia longipalpis)* but are unable to sustain *Leishmania* infections, and their presence may exert a zooprophylactic effect. We discuss environmental, physiologic, socioeconomic, and cultural factors related to chicken raising that could influence *Le. infantum* transmission in Brazilian cities and evaluate whether this practice significantly affects the risk of acquiring ZVL.

During the last 20 years, zoonotic visceral leishmaniasis (ZVL) due to *Leishmania (Leishmania) infantum* has become a serious public health problem in several Brazilian cities (1). The pathogen is transmitted by the bite of the phlebotomine sand fly *Lutzomyia longipalpis* (Lutz & Neiva 1912), and although humans can be infected, they are believed to be "dead-end" hosts; domestic dogs are the main reservoirs for the parasite. The spread and increasing prevalence of ZVL in urban areas are linked to human migrations, involving the transportation of infected dogs from ZVL-endemic regions to impoverished urban areas where *Lu. longipalpis* already exists. Although generally located on the margins of large Brazilian cities, these shanty towns (favelas) in Belo Horizonte (population 2.3 million) are dispersed throughout the urban zone, often adjacent to wealthy neighborhoods. Many of the inhabitants raise chickens, pigs, and other livestock in their yards, and because of the general climate of insecurity, keep dogs, which act as amplification hosts for *Le. infantum* (2). Thus all the factors for parasite transmission may be concentrated within a relatively small area.

The proximity of hen houses is acknowledged as a possible environmental risk factor in studies of urban ZVL (3,4), but the role chickens play in *Le. infantum* transmission has not been completely explained. A study in the Brazilian state of Bahia found that dwellings of persons with ZVL were 4.21 times as likely to have chicken houses in the yard as those whose occupants were unaffected (5), but other studies have failed to demonstrate a significant correlation. Although the attraction of chickens for *Lu. longipalpis* is indisputable, chickens, like other birds, are unable to sustain infections with *Leishmania*, and the nature of the relationship between chicken raising and ZVL is complex. In this article, we consider factors related to raising chickens that might affect transmission of *Le. infantum* in Brazilian cities and discuss whether raising chickens in urban areas could affect the risk of human acquisition of ZVL.

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Importance of Sand Fly Attraction to Chickens in *Le. infantum* Transmission

Widely differing observations regarding the degree to which *Lu. longipalpis* bites humans in different habitats, as well as the fact that female sand flies from nonanthropophilic populations can be induced to feed on humans in the laboratory indicate that this species has no strong innate host preference. Although sand flies in Brazil are known by a number of common names, including canagalinha, mosquito de palha, and asa branca, the absence of such a term to distinguish *Lu. longipalpis* from other biting flies in urban ZVL foci suggests that this fly does not constitute a substantial biting nuisance for the inhabitants.

Sand fly reproduction depends on the availability of blood meal sources such as domestic animals and synanthropic species that raid chicken houses and are potential reservoirs of *Le. infantum*, such as the fox (*Cerdocyon thous)*, opossum (*Didelphis albiventris),* and black rat (*Rattus rattus)* (2,6). Although involved in sylvatic transmission of *Leishmania,* the fox is less likely to be found in urban areas than the other two species. Host loyalty involving subpopulations of vectors would have a marked effect on *Leishmania* transmission (7). The relative attractiveness of chickens compared with other hosts at a particular site can be calculated from "forage ratios" (8), in which the percentage of sand flies feeding on the birds is divided by their relative numerical importance: values significantly >1.0

indicating selective preferences. A study in rural Colombia (9) demonstrated that *Lu. longipalpis* clearly preferred pigs and cows over chickens (values were ≤ 0.75). However, results of such studies may not be reproducible in other situations, where wind direction and the relative proximity of different hosts to host-seeking sand flies affect attraction. Comparisons of the attractiveness of different host species should also take into account differences in biomass, heat loss (a function of the surface area/volume ratio), and CO_2 production (10). Chickens produce 19–26 m^3/kg body weight of $C0_2$ per minute (11); comparable figures can be estimated as 13–17 for dogs and 8–11 for humans, when specific metabolic rate scales (in homeotherms) are used as $mass^{-0.25}$ (12). Field experiments on Marajo Island, Brazil, showed that one boy attracted significantly more female *Lu. longipalpis* than one dog or one chicken and slightly fewer sand flies than six chickens (13). Assuming that the children participating in this study each weighed about 40 kg and chickens 2 kg, then the amounts of $C0_2$ produced by one boy would be approximately 400 m^3/min, equivalent to that of about eight chickens. However, host odor is probably the most important stimulus for orientation of blood-feeding insects in open (i.e., nonforest) situations (14).

Flights of several hundred meters have been recorded for *Lu. longipalpis,* and infected sand flies attracted to an area by chicken houses may be diverted en route or displaced to other hosts. This diversion would explain the presence of (usually canine) ZVL in wealthy districts adjacent to poorer neighborhoods, a pattern seen in many Brazilian cities. Male sand flies marked with fluorescent powders traveled distances of up to 430 m between chicken houses in Montes Claros, Brazil (Kirby M. American visceral leishmaniasis—the importance of the domestic chicken *Gallus gallus* to the urban distribution of the sandfly vector *Lutzomyia longipalpis* [Diptera: Psychodidae] [M.Sc. thesis]. London: London School of Hygiene and Tropical Medicine; 2000), so such flights are clearly not limited to females in search of blood meals.

Zooprophylaxis and Factors Precluding Chickens as Hosts of *Leishmania*

Chickens have several physiologic characteristics that preclude them from sustaining *Leishmania* infections, including their body temperature of 41.0°C (15). Enzymatic processes in the sand fly function differently when triggered by different types of blood meal, and blood from certain sources may be lethal to *Leishmania* (16). Turkey blood meals significantly reduced *Le. tropica* infections in the Old World sand fly *Phlebotomus papatasi,* even when insects were infected after digestion of the blood meal, perhaps due to DNAase activity triggered by the presence of nucleated erythrocytes. A few drops of turkey blood rapidly killed *Le. tropica* promastigotes in culture, although this in vitro effect could not be the same as that in the sand fly gut and may be complement-related (17,18). Thus, not only is *Leishmania* infection unable to develop in birds, but also existing infections might be eliminated in sand flies taking a second blood meal from chickens.

For a single host species, the basic reproductive rate R_0 of a vector-transmitted pathogen is given by the following equation (19), $R_0 = \frac{ma^2bp^n}{-r \ln p}$
where m is the number of vectors per host, a is the daily biting rate of each individual vector on the host species, b is the fraction of infected vectors that actually generate infection when biting a susceptible host, p is the daily survival rate of the vectors, n is the latent period of infection in the vectors, and r is the daily recovery rate of the hosts. When host species are numerous, R_0 can be derived in general from the dominant eigenvalue of a modified “who acquires infection from whom” matrix (20). In the special case when one host (such as a chicken) is a dead end, its presence does not influence the mathematical form of eigenvalue: rather, the question is what influence this host has on a and m. The proximity of chickens to humans may potentially increase m by attracting more sand flies into the local area, or even by maintaining a higher sand fly population, through provision of additional resources. Other dead-end hosts will decrease a on humans (a zooprophylactic effect) because a given sand fly will be able to obtain its nutrition from an alternative source. Which effect dominates depends on the relative strength of these competing effects; note that a enters into R_0 as a square, compared with m, which has a linear effect. A further complication is that, if the presence of chicken houses in some areas has the effect of aggregating an existing sand fly population, an overall increase in R_0 will result (21)

Chickens as Blood Meal Sources for Maintenance of Sand Fly Populations

Although chickens cannot act as *Le. infantum* reservoirs, they may be important in maintaining vector populations and attracting mammalian reservoirs to the vicinity. Feeding success of sand flies can be measured by using the equation $G_i = Q_i/N^m_j$ (22), where G_i is the mean gain in resources (e.g., blood meal size) on host i, Q is an estimate of patch quality (in this case, number of chickens) and N^m_i, the biting rate. Nutritional quality of blood (about 90% protein by dry weight) varies between host species and G_i may also be revealed by reduced rates of development, longevity, and digestion, as well as skewed by sex ratios (14). Laboratory studies of fecundity of insects fed on blood from different hosts often fail to take into account natural factors such as host defense mechanisms (both behavioral and physiologic), activity patterns, and intra- or interspecific competition at feeding sites. Although no comparative studies of fecundity involving sand flies fed on birds exist, the mosquito *Culex pipiens* produced twice as many eggs per mg of blood when fed on canaries as when fed on humans (23). The results of this study notwithstanding, avian blood should be less nutritious than that of mammals for several reasons. Chicken erythrocytes are nucleate and have a DNA content 31 times that found in humans. They also have a lower hemoglobin content than mammalian red cells and a hematocrit value half that of mammals. These values mean

that sand flies feeding on chickens would have to ingest twice as much blood as those on mammals to obtain a meal containing the same quantity of erythrocytes. Unlike mosquitoes, sand flies do not expel any of the blood meal while feeding and cannot continue to engorge when replete (24). Even if plasma rather than erythrocytes were the essential component for ovarian development (25), total plasma protein levels in chickens are considerably lower than in dogs and pigs. In addition, catabolism of nucleic acids from chicken erythrocytes would presumably involve greater bio-energetic costs due to increased production and active transport of uric acid, the end product of nitrogen metabolism in insects (26).

Factors Favoring or Limiting the Feeding of *Lu. longipalpis* on Chicken Blood

Unlike most mammal species, chickens are inactive at night and present large areas of exposed skin on which sand flies can feed. The comb and wattles are richly supplied with capillaries but the epidermis is much thinner (~0.02 mm) on feathered areas of the body (27) and could thus be pierced more easily by the proboscis of a sand fly. Sand fly mouthparts are too short to probe deeper than the superficial loops of the host's capillaries, and the insects ingest blood from pools that form after laceration of the ends of the vessels (28). This mode of feeding exposes the female sand fly to a battery of hemostatic and inflammatory reactions, and saliva of *Lu. longipalpis* contains substances able to counteract these, including anticoagulants, apyrase to inhibit platelet aggregation and a potent vasodilator (29,30). The erythrocytes are relatively soft and easily ruptured, while the thrombocytes, which are analogous to platelets in mammals, are less efficient in reducing blood loss in birds (31). These characteristics could facilitate blood feeding by sand flies, as has been observed in triatomines (32). Reductions in blood flow rate due to colonization of the pharynx and cibarium by *Leishmania* (33) could also make feeding on chickens preferable for infected sand flies, further favoring zooprophylaxis of ZVL.

Chicken Houses as Foci of Reproductive Behavior for *Lu. longipalpis*

Male blood-sucking flies that are irregularly or widely dispersed in a habitat may gain a mating advantage by staying with the host and waiting for females to arrive (34). Male *Lu. longipalpis* encountered on a host at a particular moment usually far outnumber females, and courtship behavior involves mating aggregations or "leks" where males compete by producing sex pheromones. The effective range of the compounds involved (35) is a function of their volatility; less volatile molecules are active over shorter distances but produce a more coherent message (J.G.C. Hamilton, pers. comm.).

Preliminary trials of a pheromone-baited trap for *Lu. longipalpis* obtained better results when extracts were heated (36), and host temperature might be important in disseminating these compounds. Chickens' higher body temperature could thus favor them over mammals as lekking sites for male *Lu. longipalpis*. In view of the short effective range of male pheromones (~2 m), pheromones are unlikely to be involved in attracting sand flies to chicken houses rather than host-produced stimuli such as odor and CO_2 that extend for further distances.

Newly emerged *Lu. longipalpis* adults and larvae of several Old World species have been collected in animal shelters (37). However, attempts to recover larvae from chicken houses have been unsuccessful (38), perhaps because the nitrogen-rich feces of chickens are unpalatable to them. *Lu. longipalpis* adults may rest in chicken houses after taking blood but breed in nearby, less accessible microhabitats such as rodent burrows, where temperature, relative humidity, and light levels are more constant. Oviposition of laboratory-raised *Lu. longipalpis* involves a thigmotropic response (39), suggesting that in the wild females lay eggs in confined spaces such as crevices rather than on exposed surfaces. No evidence is available on predatory behavior by chickens toward sand flies or their natural enemies (which are largely unknown).

Interventions Focused on Chicken Houses

Chicken houses are sprayed with residual insecticides as part of the current ZVL control strategy in Brazil (40), but this spraying is constrained by costs of materials and availability of trained personnel. An alternative would be to modify the environmental factors favoring contact between vectors, reservoirs, and susceptible humans, such as proximity to chicken houses. A similar approach has been suggested for controlling dengue (41), which currently afflicts the same segment of the Brazilian population as ZVL.

With regard to conventional control programs, the relative merits of insecticidal spraying of human dwellings, chicken houses, or both, need to be considered. DDT spraying of houses in the Brazilian Amazon region failed to reduce the incidence of cutaneous leishmaniasis, perhaps because most of the vectors (*Lu. intermedia*) rested in chicken coops, which were left untreated (42). Presumably, the numbers of sand flies that did not feed on chickens were sufficiently large to balance any zooprophylactic effect, and chicken coops may only have been used as resting sites. Spraying houses alone would be an effective strategy only if all female sand flies in the vicinity could be diverted to feeding on chickens. However, spraying chicken houses alone would probably be ineffective because the odor and CO_2 produced by the birds would still attract sand flies to the vicinity, and the sand flies risk encountering infected or susceptible mammals (including humans) en route and when they rest afterwards in untreated microhabitats.

Socioeconomic Importance of Chicken Rearing

Alexander et al. (unpubl. data) found that up to 27.0% of residents of poor neighborhoods in the city of Montes Claros kept chickens for the following reasons: to produce eggs (50.0%) or meat (34.5%) for occasional personal consumption; as a hobby (23.6%); for cock-fighting (3.6%); to keep yards free of trash (9.1%); or to control scorpions (*Tityus ser-*

rulatus) (7.3%). Nevertheless, 84.6% of the people interviewed said they would stop raising chickens if it was proved that keeping chickens increased the risk of acquiring ZVL.

No information is available on the contribution of poultry products to children's nutrition in urban foci of *Le. infantum* transmission. In any case, ZVL is more likely to develop in children with moderate or severe malnutrition than in healthy children (43), and infant malnutrition is common in Brazilian cities (values of 25.9% and 19.7% are recorded for the state capitals of São Paulo and Curitiba, respectively) (44). If families derive a large proportion of their daily protein intake from chickens or eggs, prohibition of raising chickens might therefore affect the prevalence of clinical manifestations of ZVL in infected children. Current legislation that bans livestock within the limits of Brazilian cities often does not specifically prohibit poultry raising.

Conclusions

Lane (42) discussed a number of the points mentioned in this article, noting that the relationship between chicken houses and sand flies also extended to the Old World *Leishmania* vectors *Phlebotomus argentipes*, *P. langeroni, P. ariasi,* and *P. papatasi*. In Brazil several other *Lutzomyia* species also feed on chickens or at least rest in chicken houses, including the *Le. braziliensis* vectors *Lu. intermedia* and *Lu. whitmani*, so that the shelters clearly offer important man-made refuges for sand flies in urban environments. Nonetheless, the results of epidemiologic studies that attempt to incriminate chickens as a risk factor for urban ZVL are conflicting. Since the disease is potentially fatal, as is Chagas disease, zooprophylaxis as a means of control cannot be tested experimentally for ethical reasons.

The relationship between chicken raising and *Le. infantum* transmission by sand flies is summarized in the Table. Modeling the risk of *Leishmania* transmission by sand flies associated with chickens would require collecting field or laboratory data on all the factors discussed above, but current knowledge can be summarized as follows. Chicken houses attract both blood-seeking females and males seeking mates, but do not appear to act as breeding sites. They also attract potential reservoirs of *Leishmania a*nd are protected by dogs, themselves amplification hosts of the parasite. Nevertheless, chickens are refractory to *Leishmania* infection and, in certain situations, act as zooprophylactic agents. Although chicken blood may be less nutritious than that of mammals, influencing egg productivity and thus population levels of sand flies, this disadvantage

Table. Positive and negative factors associated with chicken raising that may affect the transmission of *Leishmania infantum* by *Lutzomyia longipalpis* in urban foci of zoonotic visceral leishmaniasis, Brazil

Factors affecting risk of transmission of *Le. infantum* to humans (all+)	Factors affecting risk of infection of *Lu. longipalpis* by *Le. infantum* (all-)	Factors affecting maintenance of sand fly populations (+/-)		
		Facilitation of blood feeding (+)	Nutrition (-)	Bringing sexes together (+)
Chicken rearing seen by local people as providing several benefits not directly related to L*eishmania* transmission, e.g., scorpion control, source of food and income and keeping yards free of trash	Complement levels in blood fatal to *Leishmania*?	Chicken RBCs soft and easily ruptured	Protein content of chicken plasma considerably lower than that of mammals	CO_2 and odor attractive to both males and females
Odor and CO_2 emitted by chickens attract infected sand flies to the vicinity of human dwellings	Temperature of chicken blood too high (41°C) to permit growth of *Leishmania*	Chickens easier to feed on by sand flies that have pharynx partially blocked by *Leishmania*?	Nucleated blood cells have 31 times DNA content of human erythrocytes; elimination associated w/ problems of water balance?	High chicken body temperature might favor dissemination of pheromone
Presence of chickens attracts potential reservoirs of *Le. infantum* to the vicinity of human dwellings where sand flies also present	Greater facility with which sand flies can feed on chickens would favor biting by infected sand flies whose capacity to ingest blood is compromised by blockage of the pharynx?	Chicken skin thinner than that of mammals (0.02 mm), esp. on feathered areas of body	Hematocrit value of chicken blood about 50% that of mammals; sand flies cannot concentrate blood meal during engorgement	Passivity of roosting chickens lets male sand flies display relatively undisturbed
Dogs kept to guard chicken houses from thieves and predators are themselves potential reservoirs of *Le. Infantum*	Nucleated erythrocytes in blood meal stimulate DNAase activity fatal to *Leishmania* within sand fly gut?	Thrombocytes less efficient than mammalian platelets in preventing blood loss		
Chicken houses may act as resting sites for engorged sand flies				
No evidence that chicken houses act as sand fly breeding sites although associated rodent burrows might be exploited				

[a]RBCs, red blood cells; esp., especially.

would be compensated to some extent by the greater facility with which *Lu. longipalpis* is able to feed on birds. Prohibiting chicken rearing in Brazilian cities would remove a potential source of food and income for the inhabitants of low-income neighborhoods. In fact, some health authorities currently advise householders to keep only two chickens to control scorpions, although no published data support this recommendation. A recent study modeled *Trypanosoma cruzi* transmission among populations of humans, dogs, and chickens in three Argentinian villages (45), a situation that may be considered analogous to that of urban ZVL foci in Brazil. Prevalence of infection decreased slowly as the fraction of triatomine bugs feeding from chickens increased, indicating a slight zooprophylactic effect. In addition, as the relative density of the bugs increased, the proportion that fed on humans rather than chickens decreased.

Urban ZVL is an increasingly grave public health problem in Brazil that imposes an additional strain on local health authorities and is unlikely to be resolved by current strategies. Chickens are the most common type of livestock raised in low-income neighborhoods. Understanding the role of chicken raising in the *Le. infantum* transmission cycle could lead to inexpensive and sustainable preventive measures, perhaps involving the acquiescence of local people in the removal or focal treatment of chicken houses. The role played by chickens in the epidemiology of urban ZVL clearly involves some type of balance between zooprophylaxis, maintenance of sand fly populations, and attraction of reservoir hosts of *Le. infantum*. This balance may vary in different situations but could be further clarified by the following activities: 1) field observations to determine the relative importance of chickens and other hosts as blood meal sources and lekking sites; 2) laboratory studies of comparative egg productivity of sand flies fed on chickens and other hosts; and 3) socioeconomic surveys on the importance of chickens to communities affected by ZVL in terms of income and nutrition, as well as communities' willingness to participate in preventative measures e.g., removal of chicken houses.

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EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

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

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

Why an "Emerging" Infectious Diseases journal?

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

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

What are the goals of Emerging Infectious Diseases?

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

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

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

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

Antimicrobial Resistance in *Streptococcus pneumoniae*, Taiwan

Po-Ren Hsueh*and Kwen-Tay Luh*

Taiwan has one of the highest levels of antibiotic-resistant pneumococcus in the world. Pneumococcal isolates not susceptible to penicillin first appeared in Taiwan in 1986; in 1995 an increase in the prevalence of nonsusceptibility to penicillins, extended-spectrum cephalosporins, trimethoprim-sulfamethoxazole, and macrolides as well as multidrug resistance began to be recognized. With the persistence of antibiotic selective pressure, resistance in some antibiotics reached a high plateau (β-lactam antibiotics) or continued to increase (macrolides), while novel resistance (fluoroquinolones) emerged in the last 3 years. Widespread distribution of some novel resistant 23F and 19F clones (and the international epidemic of 23F clones) contributes further to the rapid increase of resistance. Because *Streptococcus pneumoniae* is a major pathogen that causes community-acquired lower respiratory tract infections and meningitis in adults and children, antibiotic-resistance in this organism is a serious problem.

For more than a century, *Streptococcus pneumoniae* has been known as the major bacterial pathogen in humans, causing substantial illness and death (1,2). Before 1967, this organism was uniformly susceptible to penicillin. In the early 1990s, pneumococcal isolates appeared that exhibited a high level of resistance to penicillin and other β-lactam antibiotics (1). The widespread emergence of this resistance in many countries has become a major concern in recent years. The persistence of high antibiotic selective pressure in the community and international spread of epidemic or countrywide circulation of endemic multiresistant clones have substantially contributed to the crisis of resistance (3). This resistance has complicated treatment options and increased the likelihood of treatment failure (2).

The Asian region is one of the epicenters for pneumococcal resistance, and Taiwan has become the focus of pneumococcal resistance since 1996, particularly after several reports documented the alarmingly high prevalence among clinical isolates of resistance to β-lactam antibiotics and macrolides (4–12). The Center for Disease Control under the Department of Health in Taiwan established an active surveillance program in 1998 to study the epidemiologic features of invasive pneumococcal diseases in Taiwan. Furthermore, a nationwide surveillance system for antimicrobial resistance involving 12 major teaching hospitals (Surveillance from Multicenter Antimicrobial Resistance in Taiwan [SMART]) has also tracked the trends of pneumococcal resistance annually since 2000. In this report, we will discuss the trends of pneumococcal resistance, evidence of dissemination of resistant clones, and substantial community use of antibiotics, and highlight critical resistance problems.

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Disease Burden and Severity

The incidence of invasive infections caused by *S. pneumoniae* in Taiwan is still unknown, although several studies regarding invasive pneumococcal infections in adults and children have been reported (9,13–19). The overall mortality rate (42.5%) for elderly patients (≥65 years of age) with invasive infections (bacteremia, pneumonia, pleural empyema, meningitis, septic arthritis, and peritonitis) was higher than the rates for patients 19–64 years of age (22.4%) and for children (8.1%) (9). Some factors, such as the presence of serotype 3 strain, shock as initial presentation, and multilobar pneumonia, were significantly associated with death from invasive infections (14,15). Another report from central Taiwan indicated that the overall mortality rate for children with invasive pneumococcal infections was 20.3%, and in 53.3%, the infections progressed rapidly to death (18). Other studies found that 70% to 80% of adult patients with invasive pneumococcal disease had underlying diseases (malignancies, followed by congestive heart failure and diabetes mellitus) (14,15). Patients with HIV infection or multiple myeloma in whom invasive pneumococcal infection developed were extremely rare (14,13–20). No significant difference was found in the mortality rates of patients with penicillin-susceptible (PSSP) and those with penicillin-nonsusceptible *S. pneumoniae* (PNSSP) infections (9,14,15,18).

The prevalence of this organism that causes community-acquired pneumonia or meningitis in adults or children is obscure. Several reports have indicated that this organism causes 19% to 33% of infections that results in bacterial meningitis in children (21–23). In one study of adult patients, 28% of the community-acquired bacterial meningitis were caused by *S. pneumoniae*, the highest proportion after that was by *Klebsiella pneumoniae* (33%) (24). The incidence (cases per 100,000 emergency visits) of pneumococcal meningitis at

National Taiwan University Hospital was 2.8 in 1997–1998 (24). In other study, *S. pneumoniae* accounted for 21.8% of bacterial pathogens isolated from middle ear fluid from 243 children with acute otitis media (25).

Antimicrobial Resistance

Resistance to Penicillin and Other β-lactam Antibiotics

The first clinical isolate of *S. pneumoniae* not susceptible to penicillin was reported in 1986 (26). At National Taiwan University Hospital, a clinical isolate of PNSSP might have also been first seen in 1986 (the disk used for determining penicillin susceptibility was not the standard 1-µg disk). A stepwise decline in the annual rates of susceptibilities to penicillin from 1981 to 2000 at National Taiwan University Hospital (disk susceptibility data from all sites of isolates) (1) and a high prevalence (60%–84%) of clinical PNSSP isolates were noted throughout the island (disk susceptibility data from all sites of isolates) (12).

The table summarizes the results of dilution susceptibility tests for *S. pneumoniae* isolated from various clinical specimens (6–8, 10) and normally sterile sites (9) of patients seen from 1996 to 2000. The results were similar to those determined by the disk diffusion method (12). The proportions of high-level resistance to penicillin (MIC ≥2 µg/mL) varied from 8% to 33%; however, isolates exhibiting an MIC of penicillin of ≥4 µg/mL accounted for ≤12%. About 60% of isolates with intermediate resistance to penicillin also had intermediate resistance to cefotaxime or ceftriaxone, and nearly all isolates resistant to penicillin were also not susceptible to those two agents (6–9). The resistance level was higher among nasopharyngeal isolates from colonized children (71%) or among isolates from children with invasive conditions (such as bacteremia, pneumonia, meningitis, peritonitis, and empyema thoracis) than those from adults with invasive conditions (76% vs. 45%) (chi-square test, p=0.0000009) (5,9). About 50% of isolates from blood or cerebrospinal fluid samples were not susceptible to penicillin (6,7,9). Among isolates recovered from patients hospitalized in the intensive care units from five major teaching hospitals in Taiwan, 58% were not susceptible to penicillin, 33% were not susceptible to cefotaxime, and 21% showed intermediate resistance to imipenem (10).

Resistance to Macrolides

A similar trend of decreased annual rates of susceptibility for *S. pneumoniae* was also observed in erythromycin. (Table and Figure 1). Overall, the prevalence of clinical isolates of *S. pneumoniae* not susceptible to erythromycin was 67% to 100% island-wide (12). Only about one-third of the erythromycin-resistant isolates exhibited M-phenotype (27).

Resistance to Fluorquinolones or Other Antibiotics

Isolates not susceptible to ciprofloxacin (MIC ≥4 µg/mL) might have first been noted in 1996, but the first clinical isolate that was highly resistant to ciprofloxacin (MIC ≥32 µg/mL) and other newer fluoroquinolones was documented in 1999 (6,8,28,29). This isolate had mutations in genes *gyrA* and *parC* and also possessed an efflux mechanism (28). Isolates not susceptible to trimethoprim-sulfamethoxazole accounted for 60% to 90% (6–10). All isolates are susceptible to vancomycin and linezolid, but some strains were resistant to quinupristin-dalfopristin (10,29). More than 90% of PNSSP isolates were also resistant to multiple antibiotics (resistant to at least three classes of antibiotics) (6–10).

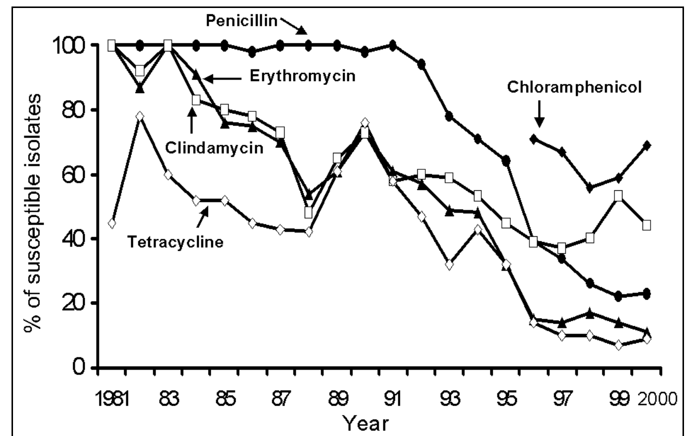

Figure 1. Prevalence of susceptibility to five antimicrobial agents for *Streptococcus pneumoniae* isolates at the National Taiwan University Hospital, 1981–2001. Susceptibility testing was performed with the disk diffusion method. For penicillin susceptibility testing, the 10-U penicillin disk was used from 1981 to 1989, and the 1-µg oxacillin disk since 1990.

Several factors limit our conclusions regarding the trends of pneumococcal resistance in Taiwan. First, data on antibiotic resistance gained from disk diffusion testing in some studies are not optimal because such studies tend to overestimate penicillin resistance compared with estimates determined by the dilution method. Second, studies that report resistance data of a mixture of invasive and noninvasive isolates and of isolates from different location for different years make it hard to assess the trends over time since resistance may vary with sites of isolates and geographic location. Third, resistance data from one hospital cannot be compared with data from larger populations since single hospitals may have more referral bias for severe cases and do not necessarily represent the phenomenon of the larger population.

Spread of Resistant Clones

Although one report has supported the idea that the international epidemic (Spanish 23F) clone was introduced and diffused in Taiwan (30), multiple domestic and novel clones (23F, 19F, and 6B), which have exhibited high-level resistance to penicillin, extended-spectrum cephalosporins, and macrolides, continuously circulate in our community (9,31,32). Two penicillin-resistant clones that acquired separate mechanisms of macrolide resistance, i.e. Taiwan-23F clone isolates exhibiting high-level resistance to erythromycin with an MIC of >256 µg/mL (*ermAM* genes mediated) and Taiwan-19F clone isolates with erythromycin resistance shown by an MIC of 1.5–8 µg/mL

Table. Summary of dilution susceptibilities for *Streptococcus pneumoniae* isolates, Taiwan, 1996–1999

	% of nonsusceptible isolates (intermediate/resistant)				
Antimicrobial agent	1996–1997[a] (n=200)	1996–1997[b] (n=550)	1998–1999[c] (n=267)	1998–1999[d] (n=288)	2000[e] (n=24)
Penicillin	61 (28/33)	56 (43/13)	76 (51/25)	56 (34/22)	58 (50/8)
Amoxicillin (-clavulanate)		50 (34/16)	33 (32/1)	22 (12/10)	—
Cefuroxime	—	—	67 (16/51)	43 (6/37)	—
Ceftriaxone (cefotaxime)	39 (16/23)	13 (11/2)	56 (54/2)	25 (15/10)	33 (29/4)
Cefepime	43 (19/24)	—	—	—	42 (21/21)
Imipenem	—	15 (13/2)	—	14 (14/0)	21 (21/0)
Meropenem	—	—	—	—	4 (4/0)
Erythromycin	83 (6/77)	74 (5/69)	—	76 (4/74)	—
Azithromycin	—	78 (4/74)	94 (4/90)	78 (6/72)	—
Clarithromycin	90 (9/81)	—	95 (6/89)	—	—
Trimethoprim-sulfamethoxazole	87 (6/81)	—	65 (33/32)	71 (30/41)	—
Ciprofloxacinf	2	—	4	—	0
Levofloxacin		—	1 (0/1)	—	0
Moxifloxacin	0	—	1 (0/1)	—	0
Rifampin	0	7 (7/0)	—	—	—
Vancomycin	0	0	—	0	0
Quinupristin-dalfopristin	—	—	8 (6/2)	—	42 (38/4)
Linezolid	—	—	0	—	0

[a]Data adopted from reference 6. All isolates were recovered from various clinical specimens (60% of isolates from respiratory secretions and 27% from blood samples) of patients treated at the National Taiwan University Hospital (NTUH).
[b]Data adopted from references 7. All isolates were recovered from various clinical specimens (36.7% from sputa and 42.3% from sterile sites) of patients treated at 14 major hospitals in Taiwan.
[c]Data adopted from references 8. All isolates were recovered from various clinical specimens, including 86.4% from respiratory secretions and 8.8% from sterile sites (cerebrospinal fluid, blood, ascites and pleural fluid) of patients treated at five teaching hospitals in Taiwan.
[d]Data adopted from reference 9. All isolates were recovered from normally sterile sites (cerebrospinal fluid, blood, ascites and pleural fluid) of patients treated at all major hospitals in Taiwan.
[e]Data adopted from reference 10. All isolates were recovered from various clinical specimens (12 isolates were recovered from non-respiratory secretions) of patients treated at five teaching hospitals in Taiwan.
[f]Ciprofloxacin MIC ≥4 µg/mL.

(*mefE* gene–mediated), have already spread (30). Isolates belonging to the same clone caused various invasive diseases of different patients who lived in different parts of Taiwan, and resulted in bacteremic pneumonia in siblings (9,31,33). The spread of an endemic and highly resistant 23F clone (penicillin MIC, 4 µg/mL and erythromycin MIC, >256 µg/mL) in one day care center was also reported (34). The spread of Taiwan clones to other parts of the world (Taiwan 19F-14 and Taiwan-6B clones to Hong Kong and United Kingdom; Taiwan 19F-14 to the United States) has also been documented (30,35,36). Although the dissemination of fluoroquinolone-resistant strains has been documented in Hong Kong and Brooklyn, New York (37,38), to date, the clonal spread of these resistant strains has not been found in Taiwan.

Impact of Resistance on Antimicrobial Therapy

A high dose of intravenous penicillin G is still recommended as one of the primary drugs of choice for the empirical treatment of patients with community-acquired pneumonia who need hospitalization because about 10% of our clinical isolates have an MIC of penicillin of ≥4 µg/mL (6–10,39). MIC testing, mostly by the E-test, for penicillin-nonsusceptible (oxacillin 1-µg disk) isolates recovered from respiratory secretion or blood specimens from patients without meningitis, is always performed in most teaching hospitals in Taiwan (39, 40). Obviously, the guidelines for the treatment of community-acquired pneumonia in adults recommended by the American Thoracic Society in 2001 (41) and the Infectious Diseases Society of America in 2000 (42), as well as suggestions for the management of sinusitis, otitis media, and chronic bronchitis, which include one macrolide alone as the drug of choice or as an alternative antimicrobial agent, are inappropriate in Taiwan. This is because of the high incidence of macrolide resistance and the high proportion of MLS_B-phenotype among these resistant isolates.

Serotype Prevalence and Vaccine Coverage

Figure 2 illustrates the distribution of six major serogroups or serotypes of *S. pneumoniae* isolates from 1984 to 1998 (4,6,7,9,19,43). The frequencies of serogroups (serotypes) 23

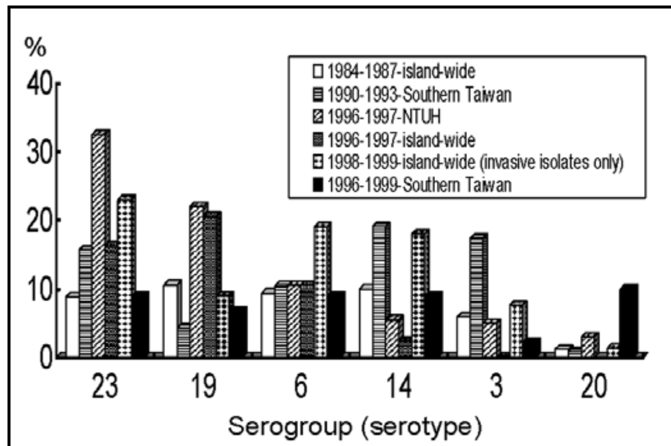

Figure 2. Distribution of six major serogroups or serotypes of clinical isolates of *Streptococcus pneumoniae*, Taiwan, 1984–1998.

(23F) and 19 (19F) increased remarkably, whereas those of serotypes 14, 3, and 1 declined. Some unusual serotypes (serotypes 20, 11, 7, and 8) appeared to emerge in southern Taiwan. No relationship was found between the serogroup or serotype distribution of isolates and the patients' ages ($p > 0.05$) (6,7,9). PNSSP exhibited resistance to isolates from various serogroupsor serotypes. Among them, serogroups 11 and 23 isolates had the highest incidence of penicillin nonsusceptibility and multidrug resistance. However, serotype 3 isolates (0%–22%) and serotype 20 (11%) isolates had lower rates of nonsusceptibility to penicillin (4,6,9,19). In general, more than 90% of isolates causing invasive infections were included in the serogroups or serotypes covered by the 23-valent pneumococcal and 7-valent conjugate vaccine (4,9).

Extent of Antibiotic Use in the Community

Before 1995, few regulations regarding antibiotic use existed for physicians in primary care clinics or hospitals. Furthermore, many antibiotics could easily be obtained at drugstores without a prescription. Although the medical payment has been regulated by the National Health Insurance program, implemented in Taiwan in 1995, antibiotics are still commonly used and seem to be overprescribed in primary care units (44). From 1996 to 1999, about 12% to 14% of total patient-visits in primary care units had antibiotic use. The common cold (32.3%) was the most frequent diagnosis for which antibiotics were prescribed. Penicillins (35.4%), cephalosporins (26.5%), and macrolides (21.6%) were the most commonly prescribed classes of antibiotics (44). In 1999, a reported point prevalence rate of about 7.5% among healthy high school students and healthy ambulatory elderly persons who had antimicrobial activity in their urine suggested that this may be the baseline for antibiotic use in the community (45). Surprisingly, over half of the patients who came to the emergency department of a large teaching hospital in Taiwan had taken antibiotics within the previous 12–48 hours. With the increasing and highly selective pressure of antibiotic usage in our community, the crisis of resistance continues to exist.

In the new millennium, the Center for Disease Control under the Department of Health in Taiwan has made nationwide surveillance of antimicrobial resistance and strict control of antibiotic usage major tasks. The new regulations for antibiotic prescription, established by the Bureau of National Health Insurance in 2001 (44), restrict the inappropriate use of the so-called first-line antibiotics (first-generation cephalosporins, macrolides, and gentamicin) for treating various infections, particularly the trivial upper respiratory tract infection. The use of the 23-valent pneumococcal vaccine, especially among high-risk adults and older persons, is encouraged, and new protein-conjugate vaccines will be introduced in the near future.

Conclusion

Taiwan has one of the highest levels of antibiotic resistant pneumococcus in the world. With the increase in international travel, the interchange of resistant clones among countries is unavoidable and the widespread distribution of these clones is expected. Strategies to limit the upsurge of resistant pneumococcus include improved surveillance, reduced antibiotic usage, and increased vaccination of persons at high risk. The judicious use of antimicrobial agents is necessary to avoid providing a selective advantage for resistant organisms. The active surveillance of resistant organisms can help track local resistance problems, prevent the future dissemination of these organisms, and provide options appropriate for empiric therapy.

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Isolation and Genetic Characterization of *Rift Valley fever virus* from *Aedes vexans arabiensis,* Kingdom of Saudi Arabia

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An outbreak of Rift Valley fever in the Kingdom of Saudi Arabia and Yemen in 2000 was the first recognized occurrence of the illness outside of Africa and Madagascar. An assessment of potential mosquito vectors in the region yielded an isolate from *Aedes vexans arabiensis,* most closely related to strains from Madagascar (1991) and Kenya (1997).

On September 10, 2000, accounts of unexplained hemorrhagic fever in humans and associated illness in livestock along the southwestern border of Saudi Arabia and neighboring Yemen were reported to the Ministry of Health, Kingdom of Saudi Arabia. On September 15, 2000, the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, confirmed a diagnosis of Rift Valley fever in serum samples submitted by the Ministry of Health. This confirmation marked the first occurrence of Rift Valley fever outside of Africa (1).

Rift Valley fever virus (RVFV) is an important veterinary pathogen in Africa causing abortions and deaths in young animals, primarily goats and sheep (2). This mosquito-borne virus can also infect humans by arthropod blood-feeding or by contact with infected animal fluids and tissues. RVFV infection in humans is generally not apparent or is self-limiting; serious complications including hemorrhagic fever, encephalitis, and retinitis can occur. Vector-borne virus transmission in Africa is generally associated with periodic heavy rainfall during epizootics and attendant human infections (2).

We conducted an entomologic investigation in the Asir, Jizan, and Makkah Regions, north of the suspected origin of the outbreak in Jizan, and in neighboring Yemen. Because this outbreak in the Arabian Peninsula was the first recorded outside of Africa, we wanted to determine the potential arthropod vectors and their larval habitats. A review of recent human infections indicated that the affected households were located in the foothills and at the base of the Sarawat Mountains. Four locations were selected for arthropod collections, Muhayil, Al Birk, Rijal Alma'a, and Al Majardah, because these areas are representative of the different ecologic habitats from coastal plain to mountainous regions. This fertile plain used for cultivation is known as the Tihamah.

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The Study

We collected adult and immature arthropods on December 5–13, 2000, using carbon dioxide–baited CDC miniature light traps and by sampling potential larval habitats with dippers. Adult specimens were frozen in liquid nitrogen for later virus testing in Fort Collins, Colorado. To investigate potential vertical transmission of RVFV, mosquito larvae were reared to adults in the malaria control laboratory in Abha, Asir Region, for later virus testing in the United States.

All collection sites were in very arid habitats; the soil is dry and rocky, and *Acacia* species are the only trees present. Livestock, including goats (the predominant animal), sheep, camels, and cattle, were present at every site. In general, livestock are housed at night very close to the owners' homes. Light traps were hung in and near the residences of recent patients, and the area was examined for larval habitats. Typical larval habitats included wastewater catchments from houses that yielded mainly *Culex pipiens* complex mosquitoes; pools at the edge of wadis; and small, walled, passively or actively flooded cultivated plots that yielded *Aedes vexans arabiensis* and *Ae. vittatus* (3). *Ae. (Stegomyia) unilineatus,* a mosquito species previously recorded from Africa, India, and Pakistan, was found in light trap collections from several sites (Godsey MS, submitted for publication). The immature and adult arthropod collections are presented in Table 1. The low species diversity and small numbers collected in light traps may have reflected that rainfall was light to nonexistent 2 weeks before our collection efforts. We did examine one walled farming plot, which contained water to a depth of approximately 5 cm; it held enormous numbers of *Ae. vexans arabiensis* larvae and pupae. Overnight, this habitat had dried up to a 2-m diameter pool.

Collected arthropod specimens were identified and placed into pools of up to 50 individual mosquitoes by collection site. A total of 161 pools were triturated, clarified by centrifugation, and spread onto confluent sheets of Vero cells in six-well plates (4). A single pool (SA01 #1322) yielded a virus isolate. The virus was identified as RVFV by sequencing a DNA fragment amplified from the M segment by reverse transcription-polymerase chain reaction using the primers RVF3082 5′actttgtgggagcagccgtatctt3′ and RVF3400 5′cctgcttcccgcct atcatcaaat3′.

RVFV was isolated from a pool of 37 *Ae. vexans arabiensis* female mosquitoes collected by light trap at site 3 (N18° 45.089 min; E41° 56.373 min) near the city of Muhayil. A human infection was recorded from this site ("Agida"); we also witnessed aborted bovine fetuses on the property. Other

Table 1. Arthropods collected in the Kingdom of Saudi Arabia, December 5–13, 2000

Taxon	Female	Male
Specimens reared from larvae and pupae		
Aedes vexans arabiensis	887	858
Ae. vittatus	0	2
Culex pipiens complex	67	47
Specimens collected in carbon dioxide–baited light trap		
Anopheles dthali	50	0
Ae. vexans arabiensis	122	0
Ae. vittatus	6	3
Ae. aegypti	2	0
Ae. unilineatus	18	3
Cx. pipiens complex	266	149
Cx. nebulosus	1	0
Cx. salisburiensis	1	0
Cx. tritaeniorhynchus	42	0
Aedes species	16	0
Anopheles species	142	1
Culex species	31	0
Psychodidae	61	18
Ceratopogonidae	26	3

arthropods collected at this site included *Cx. tritaeniorhynchus* and psychodid sandflies.

RVFV is a member of the virus family, *Bunyaviridae*; the genomes of these viruses exist in three pieces or segments: small (S), medium (M), and large (L). We sequenced a portion of each of the genome segments and analyzed them by the maximum likelihood algorithm in PAUP (5) in relation to the published sequences of other geographic isolates to determine the possible origin of the mosquito isolate and whether the isolate was a possible reassortant between two existing virus strains (5,6). Maximum likelihood trees for each genomic segment shared identical topologies (data not shown). The congruence of placement of the Saudi virus strain in the three trees indicated that this virus was not a reassortant. The phylogram for segment M (Figure; Table 2) demonstrates that the most closely related RVFV isolates were from Kenya (1997) and Madagascar (1991). Reasonable hypotheses to explain how RVFV was introduced into the Kingdom of Saudi Arabia (and/ or Yemen) from East Africa are that an infected mosquito was carried over the narrow waterway between the Red Sea and the Gulf of Aden by air currents or that infected livestock were imported from East Africa. How long the virus was in the Arabian Peninsula before the epidemic occurred is unknown.

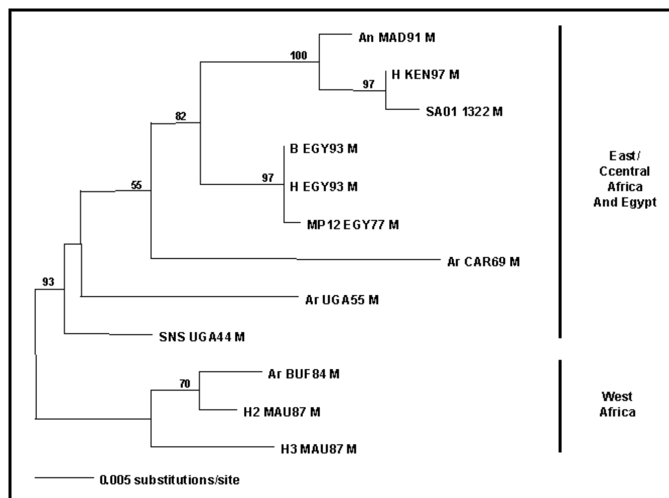

Figure. Maximum likelihood phylogram of African *Rift Valley fever virus* strains (see Table 2) and mosquito isolate from the Kingdom of Saudi Arabia based on a 655-bp DNA fragment from the M segment (4).

The most abundant culicine mosquitoes we collected were *Ae. vexans arabiensis*, *Cx. pipiens* complex, and *Cx. tritaeniorhynchus*. All three species should be considered important epidemic and epizootic vectors of RVFV in Saudi Arabia. The floodwater mosquito, *Ae. vexans arabiensis*, has the potential to be an important epidemic and epizootic vector because of the tremendous numbers of individual mosquitoes that are

Table 2. *Rift Valley fever virus* strains used in the phylogenetic analyses

Virus designation	Strain name	Geographic origin	Year of isolation	Source
SNS UGA44	Smithburn	Uganda	1944	Entebbe strain
Ar UGA55	Lunyo	Uganda	1955	Mosquito
Ar CAR69	Ar B 1976	Central African Rep.	1969	Mosquito
MP12 EGY77	MP12	Egypt	1977	ZH 548 strain
Ar BUF84	Ar D 38457	Burkina Faso	1984	Mosquito
H2 MAU87	H D 47311	Mauritania	1987	Human
H3 MAU87	H D 47408	Mauritania	1987	Human
An MAD91	An Mg 990	Madagascar	1991	Bovine
B EGY93	B EGY 93	Egypt	1993	Buffalo
H EGY93	H EGY 93	Egypt	1993	Human
H KEN97	384-97.1	Kenya	1997	Human
Ar SA01	SA01 1322	Saudi Arabia	2001	Mosquito

produced after a flooding rain. Whether or not RVFV is able to persist on the Arabian Peninsula is unknown. Clearly, vertical transmission of the virus in the epidemic mosquito vector would be an important factor to consider.

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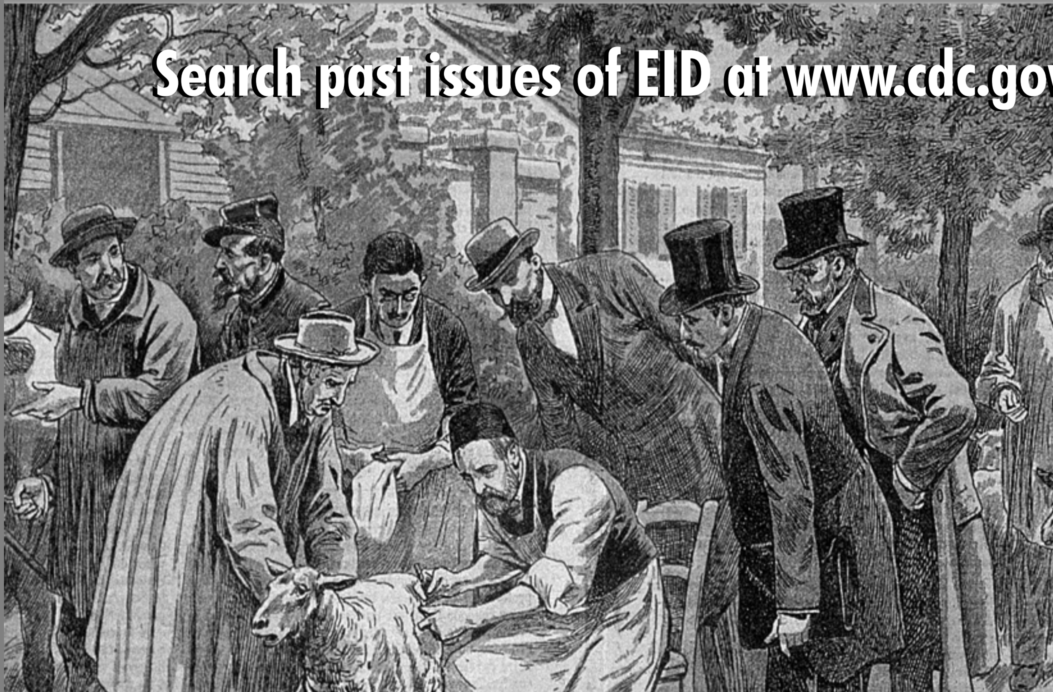

Rat-to-Human Transmission of Cowpox Infection

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We isolated *Cowpox virus* (CPXV) from the ulcerative eyelid lesions of a 14-year-old girl, who had cared for a clinically ill wild rat that later died. CPXV isolated from the rat (*Rattus norvegicus*) showed complete homology with the girl's virus. Our case is the first proven rat-to-human transmission of cowpox.

A 14-year-old girl was admitted to the hospital with ulcerated nodules on her upper lip and her left lower eyelid and several molluscum-like-lesions on her right eyelids (Figure 1). She was feverish but otherwise in good health. Values of complete blood and differential counts were within normal limits. The erythrocyte sedimentation rate was 41 mm the first hour, and she was treated orally with ciprofloxacin. Within a few days the lesions on her eyelids developed into crater-like ulcers, which were surrounded by inflammatory tissue and were later covered by thick black crusts; and sedema and erythema developed on the right side of her face. At home she kept turtles, hamsters, guinea pigs, birds, ducks, cats, and a dog. She also cared for a clinically ill wild rat, which she had found 2 weeks before admission. After 6 days of care, the rat died and was buried. During the following 4 weeks, the ulcerated lesions on the girl's face healed and left atrophic scars.

Routine virus isolation procedures from biopsies of the eyelid lesions in Vero cells showed the presence of *Orthopoxvirus*. Serum immunoglobulin (Ig) M antibodies to the viral isolate and to *Vaccinia virus* were detected upon admission. The virus was identified as *Cowpox virus* (CPXV) by polymerase chain reaction (PCR) and sequence analysis (PCR directed at the *Orthopoxvirus* fusion protein gene, as described by Chantrey et al.) (1). The rat, identified as *Rattus norvegicus,* was recovered for laboratory testing. CPXV was isolated from brain samples from the rat, and a swab was taken from its paw. Sequence analysis of the rat's virus indicated complete homology with the girl's virus. As shown in Figure 2, the sequence analysis clearly demonstrates that the virus was CPXV.

Human CPXV is a rare zoonosis, which is transmitted as a result of contact with animals.

Usually localized at the site of inoculation, CPXV lesions progress from a papule through vesiculation and pustulation into an ulcerative nodule, which is covered with an elevated border with a black eschar. Ulcera heal with scar formation. Differential clinical diagnosis of CPXV includes herpes virus infection, anthrax, and orf (caused by a *Parapoxvirus*). CPXV is almost always a self-limiting disease in immunocompetent hosts.

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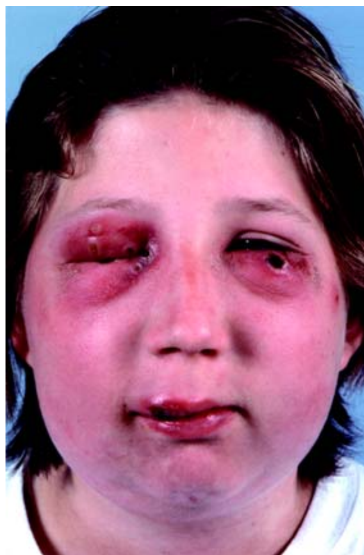

Figure 1. Cowpox lesions with ulcerated nodules on upper lip and left lower eyelid, several molluscum-like-lesions on the right eyelids, and oedema and erythema of the right side of the face.

Although wild rodents are the reservoir hosts of CPXV (1), transmission to humans has only been described from accidental hosts such as infected cats, cows, and animals in zoos and circuses (2–4). Circumstantial evidence of rodents as source of infection has been reported in two human cases: one infection was associated with a suspected rat bite (5); the other infection was in a person who cared for a sick wild field mouse (6). Person-to-person transmission has not been reported.

Our case is the first proven wild rodent-to-human transmission. Serologic surveys have shown that CPXV is a widespread endemic infection in European wild rodents with the

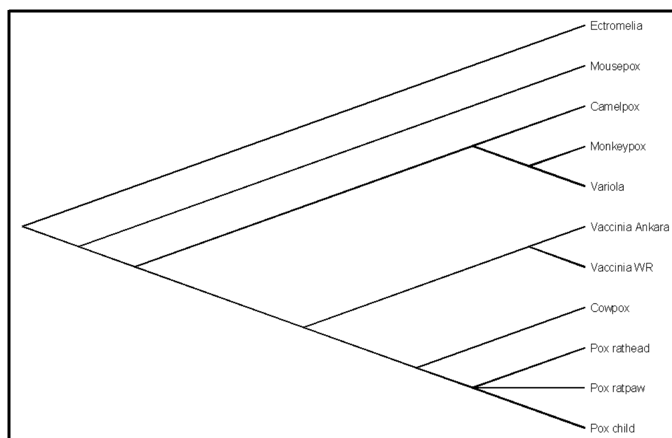

Figure 2. Phylogenetic tree of nucleotide sequences of 163-bp *Orthopoxvirus* fusion gene amplicons from the patient and rat (head and paw), *Cowpox virus*, *Vaccinia virus* (strain Ankara and WR), *Camelpox virus*, *Monkeypox virus*, *Variola virus*, and *Ectromelia virus* (*Mousepox virus*). The nucleotide sequences were aligned by using BioEdit software package (T. Hall, Dept. of Microbiology, Raleigh, NC). Phylogenetic relationships were determined by using the Lasergene software packages (DNASTAR Inc., Madison, WI).

highest seroprevalence in bank voles (*Clethrionomys glareolus*), wood mice (*Apodemus sylvaticus*) and field voles (*Microtus agretis*) (1). Seroprevalence rates in rats are not available; therefore, whether rats might also be reservoir hosts or act as liaison (accidental) hosts is unclear. Since smallpox has not occurred naturally anywhere in the world since 1977, immunization with *Vaccinia virus* has been discontinued, which has led to a declined cohort immunity to orthopoxviruses including CPXV, and thus may result in an increased incidence of human CPXV infections (2,7). Physicians must be aware that zoonosis may not only be contracted from accidental hosts (e.g., cats and cows) but also directly from a primary natural reservoir (rodents).

Dr. Wolfs is an infectious diseases pediatrician at the Wilhelmina Children's Hospital, Utrecht, the Netherlands. His research interests include HIV and the interaction between viral and bacterial infections.

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Naturally Occurring *Ehrlichia chaffeensis* Infection in Two Prosimian Primate Species: Ring-tailed Lemurs (*Lemur catta*) and Ruffed Lemurs (*Varecia variegata*)

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A naturally occurring infection of *Ehrlichia chaffeensis* in lemurs is described. DNA of *Ehrlichia chaffeensis* was identified by polymerase chain reaction in peripheral blood from six of eight clinically ill lemurs. Organisms were cultured from the blood of one lemur exhibiting clinical and hematologic abnormalities similar to those of humans infected with *E. chaffeensis.*

Phylogenetically, *Ehrlichia* species comprise an obligate intracellular group within the alpha *Proteobacteria* (1). A recent proposal by Dumler et al. resulted in reclassification of the members of the three *Ehrlichia* serogroups among the genera *Ehrlichia, Anaplasma*, and *Neorickettsia* (2). In addition to causing disease manifestations in humans and several domestic animal species, various *Ehrlichia* and *Anaplasma* species can be found in a wide range of wild animals that, in many instances, compose the blood reservoir from which ticks acquire infection for subsequent transmission to other mammals (3).

Nonhuman primates have been experimentally infected with *E. canis* or *E. equi* (recently reclassified as *A. phagocytophila*)(4–6), but, to our knowledge, natural infection of a nonhuman primate with an *Ehrlichia* or *Anaplasma* species has not been reported previously. We describe an epizootic of *E. chaffeensis* infection in a lemur colony, located in Durham, North Carolina.

The Outbreak

Lemurs were housed at the Duke University Primate Center in two extended family groups of 9 and 10 animals, respectively. During the months of October 2000 through April 2001, lemurs were housed in wire-enclosed cages averaging 22 x 24 x 8 ft with access to indoor, heated rooms. On May 1, 2001, both groups were released into a 22.5-acre, fenced, mixed pine and deciduous hardwood forest in Durham for the summer.

From May 16 to June 25, 2001, anorexia, fever, lethargy, and lymphadenopathy developed in seven ring-tailed lemurs (*Lemur catta*) and one red ruffed lemur (*Varecia variegata rubra*), ranging in age from 14 months to 17 years. *Amblyomma americanum*, *Rhipicephalus sanguineus,* and *Dermacentor variabilis* adult ticks were found on lemurs at the time of illness, but the numbers of ticks were not quantified.

At the onset of illness, animals received a physical exam at which time blood was drawn for complete blood counts and serum chemistry profiles. Additional EDTA-anticoagulated peripheral blood was stored at –80°C for subsequent DNA isolation and polymerase chain reaction (PCR) amplification. Two milliliters of EDTA-anticoagulated blood were maintained at room temperature for attempted isolation of *Ehrlichia* organisms. Urine was collected either as a voided midstream sample or by cystocentesis. Specimens collected from inguinal lymph nodes by fine needle aspiration were submitted to a commercial laboratory for evaluation by a cytopathologist. Peripheral blood smears and lymph node aspirates were stained with a Wrights-Giemsa stain and evaluated by light microscopy.

Because of the lack of published comprehensive normal blood values for ring-tailed lemurs, complete blood counts and serum chemistry profile results obtained from 18 clinically healthy ring-tailed lemurs that had undergone routine physical examinations at the Duke University Primate Center during the years 1995 through 2000 were used for comparison to values obtained from the *E. chaffeensis*–infected lemurs. As normal clinical pathology values for red ruffed lemurs vary minimally from that of normal ring-tailed lemurs described here, data from the eight ill animals were analyzed as a single group rather than as separate species (7).

Thrombocytopenia was the most commonly observed hematologic abnormality, followed by lymphopenia, leukopenia, and neutropenia. Hematocrit values were normal to elevated in all animals (Table). Hyperbilirubinemia in the presence of low to normal serum alkaline phosphatase values was the most common biochemical abnormality, followed by azotemia (increased urea nitrogen and creatinine), hyponatremia, hypochloremia, hyperglycemia, mild increases in serum transferase activities, and hypoproteinemia with corresponding hypoalbuminemia. Proteinuria (urine dipstick values ranging from 2+ to 3+) was detected in five of seven lemurs in which urinalysis was performed. Protein/creatinine ratios ranged from 0.4 to 1.2. Urine protein/creatinine ratios in lemurs without detectable protein by urine dipstick examination ranged from 0.1 to 0.2 (data not shown). Morulae were seen in lymphocytes and monocytes in lymph node aspirates from three of eight clinically ill lemurs (Figure). Morulae were not seen in leukocytes in peripheral blood smears, although examinations of buffy coat smears were not performed.

DNA extraction was performed with commercially available QIAmp Blood kit (Qiagen, Chatsworth, CA) from 200 µL

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Table. Comparison of blood parameters from normal and *Ehrlichia chaffeensis*–infected lemurs[a]

Test	Normal (n=18)			Infected (n=8)		
	Mean	SD[b]	Range	Mean	SD	Range
Glucose (mg/dL)	127	55	55–238	226	88	96–337
Urea nitrogen (mg/dL)	20	5	8–29	38	14	15–57
Creatinine (mg/dL)	0.9	0.1	0.7–1.2	1.7	0.2	1.2–1.9
Total protein (g/dL)	7.8	0.3	7.1–8.2	7.0	0.9	5.3–8.1
Albumin (g/dL)	5.7	0.3	5.2–6.6	5.0	0.7	3.8–6.0
Total bilirubin (mg/dL)	0.4	0.1	0.3–0.6	1.0	0.2	0.6–1.3
Alkaline phosphatase (IU/L)	240	83	125–476	89	38	44–144
Aspartate transferase (IU/L)	46	26	20–128	86	41	33–155
Alanine transferase (IU/L)[c]	81	51	10–210	128	85	47–321
Sodium (mg/dL)	146	4	141–152	137	3	134–142
Chloride (mg/dL)	104	4	97–111	97	4	91–103
Platelets ($x10^3/\mu L$)	327	132	165–685	181	135	34–410
Packed cell volume (%)	51.1	3.6	45.6–57.6	55.1	5.9	46–62.4
WBC ($x10^3/\mu L$)[d]	6.1	1.5	3.9–8.8	5.0	2.9	1.0–9.5
Neutrophils ($x10^3/\mu L$)[d]	3.6	1.3	0.3–6.0	2.8	1.9	0.3–6.3
Lymphocytes ($x10^3/\mu L$)	2.7	1.5	1.1–5.4	1.6	1.0	0.2–3.0

[a]Variation between means of normal and infected animals is significant at a confidence level of $p<0.05$ using the 2-sample t test, except where otherwise specified.
[b]SD, standard deviation.
[c]$p=0.18$.
[d]$p=0.34$; WBC, leukocyte count.

EDTA blood samples that had been frozen at –80°C. PCR was performed in a two-step method as previously described (8), first using primers specific for the genera *Ehrlichia* and *Anaplasma* and then using primer pairs specific for *E. canis, E. chaffeensis, E. ewingii,* and *A. phagocytophila,* on all genus-positive samples. Culture-grown *E. chaffeensis* was used as a positive control. *E. chaffeensis* DNA was amplified from blood samples from six of eight affected animals. Coinfections with multiple *Ehrlichia* spp. were not found.

Leukocytes obtained from a clinically ill red ruffed lemur were isolated and cultured in DH82 cells at 37°C with 5% CO_2 as described (9). After 16 days in culture, many morula-like inclusions were visible on stained cytospin preparations. A postinoculation day 16 sample of the cultured cells was also processed for PCR amplification. Primers specific for *E. chaffeensis* yielded an amplicon of the appropriate size.

The clinical condition of animals improved rapidly after treatment with doxycycline, 5 mg/kg orally twice daily, was initiated. Improvement in the degree of fever and anorexia were noted as early as 24 hrs after the start of treatment in all animals. Time to complete resolution of clinical symptoms varied depending on the severity of clinical signs, but all lemurs appeared behaviorally normal by day 7 of therapy. Similarly, hematologic values were normal for the seven lemurs in which laboratory tests were repeated 12–87 days after doxycycline therapy was completed.

Conclusions

An unanticipated series of events created the opportunity for an epizootic of *E. chaffeensis* infection involving lemurs, as described in this report. In association with fence maintenance and construction during the winter, the inadvertent introduction of several white-tailed deer into the lemur's 22-acre summer enclosure facilitated the transport of ticks onto the facility. In addition to *A. americanum*, *R. sanguineus* and *D. variabilis* ticks were removed from the lemurs at the time of illness. *R. sanguineus*, the brown dog tick, most often feeds selectively on dogs and is the vector for *E. canis* infection. *D. variabilis*, the common dog tick, is known to feed on deer as well as many other small to medium mammals and is most commonly associated with Rocky Mountain spotted fever. *Amblyomma americanum* is considered the most important vector tick for transmission of *E. chaffeensis,* and deer are an important wildlife reservoir for *E. chaffeensis* in nature (10,11). Considered a very aggressive tick species, it will feed on numerous wild and domestic animals, as well as humans. In conjunction with the substantial increase in the deer population in the southeastern United States, human and animal exposure to *A. americanum* has increased dramatically, coincident with a gradual northern expansion of the range of this tick species, particularly in the eastern and central United States (1,10). As a result, the incidence of human ehrlichiosis has increased dramatically (12). Similar to *Rickettsia rickettsii*, the cause of Rocky Mountain spotted fever, *E. chaffeensis* causes

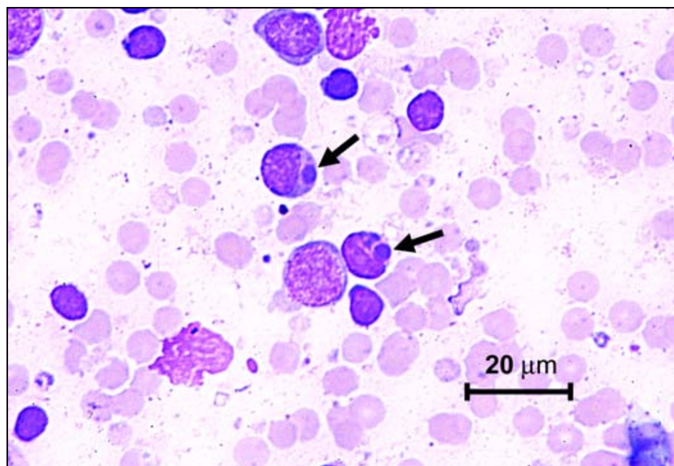

Figure. Photomicrograph of a lymphocyte from a lymph node aspirate containing *Ehrlichia* morula (arrow). Stain is with Wright's-Giemsa.

substantial illness and annual case-fatality rates that range from 5% to 10% of reported human cases (1).

The inadvertent exposure of lemurs to *E. chaffeensis* resulted in clinical and hematologic abnormalities comparable to those reported in dogs (8) and humans; these abnormalities included thrombocytopenia, neutropenia, leukopenia, and lymphopenia (13,14). Similar to humans, *E. chaffeensis*–infected lemurs developed mild hyperbilirubinemia and increases in serum aminotransferase levels. Similar to dogs experimentally infected with *E. canis*, lemurs developed proteinuria, which resolved after therapeutic elimination of *E. chaffeensis* (15). Despite anorexia and accompanying dehydration, which should result in hypernatremia and hyperchloremia, serum sodium and chloride values were below the reference range in six of seven ill lemurs. Although reported in individual cases (14), hyponatremia and hypochloremia are not typically associated with human *E. chaffeensis* infections (12,13). Hyperglycemia in the affected animals was attributed to stress associated with restraint in connection with the concurrent illness.

Previous reports of experimental infections of nonhuman primates with *Ehrlichia* or *Anaplasma* suggest that susceptibility may vary with the species of primate infected as well as the infective agent**.** In 1936, Donatien and Lestoquard reported severe disease following experimental infection of long-tailed macaques (*Macaca fasicularis)* with *E. canis,* whereas infection of vervet monkeys (*Cercopithecus pygerythrus*) did not induce disease (5). Neutrophilic morulae, fever, and anemia were observed in rhesus macaques (*Macaca mulatta)* and baboons (*Papio anubis*) experimentally infected with *A. phagocytophila,* but behavioral abnormalities were not observed (4). In a more recent study, two rhesus macaques experimentally infected with *A. phagocytophila* developed pyrexia, lethargy, neutropenia, thrombocytopenia, anemia, and morulae in monocytes and neutrophils (6). Collectively, these reports indicate that disease manifestations can develop in nonhuman primates when they are infected with *E. canis, A. phagocytophila,* or *E. chaffeensis.*

In summary, after transmission of *E. chaffeensis,* presumably by *A. americanum*, disease manifestations very similar to those reported in human patients developed in lemurs in a research colony. For diagnosis, morula may be found in monocytes and lymphocytes in lymph node aspirates. *E. chaffeensis* can be isolated in tissue culture and *E. chaffeensis* DNA can be amplified from peripheral EDTA anti-coagulated blood samples. Treatment with doxycycline, the drug of choice for treating human and canine ehrlichiosis, elicits a dramatic clinical and hematologic response. The impact of vector-borne diseases should be considered when working with nonhuman primate colonies maintained in natural environments in tick-endemic areas.[1]

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[1]Approximately 1 year after the outbreak in this report, *E. chaffeensis* was amplified by PCR from an ill lemur in Virginia that was unrelated to the Duke colony. The clinical and hematologic manifestations were consistent with those seen in lemurs in this report.

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EMERGING INFECTIOUS DISEASES

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Increasing Fluoroquinolone Resistance in *Campylobacter jejuni*, Pennsylvania, USA,1982–2001[1]

Irving Nachamkin,* Huong Ung,* and Ming Li*

Fluoroquinolone-resistant *Campylobacter jejuni* has been observed worldwide and is now being seen in the United States. Among patients in our health-care system in Pennsylvania, fluoroquinolone-resistant *C. jejuni* were not observed from 1982 to 1992; however, resistance increased to 40.5% in 2001. Resistance to erythromycin remains at a low level (<5%).

Campylobacter jejuni is the most common cause of bacterial gastroenteritis in the United States, where an estimated 2.5 million cases occur each year (1). *Campylobacter* enteritis is primarily a foodborne illness; poultry is the major source for human infection (1). Most campylobacter infections need not be treated with antimicrobial agents; however, fluoroquinolones have been commonly used to treat serious *Campylobacter* infections and are also used as empiric therapy for travelers' diarrhea (2).

Fluoroquinolone-resistant *C. jejuni* was recognized during the late 1980s in Europe, where researchers suggested that such resistance was due, in part, to acquisition of fluoroquinolone-resistant strains from animal sources (3). Smith and colleagues recently reported fluoroquinolone-resistant *C. jejuni* in Minnesota and found that, from 1992 to 1998, fluoroquinolone resistance increased from 1.3% to 10.2% (4). Recent data from the National Antimicrobial Resistance Monitoring System (NARMS) show that 14.2% of isolates submitted to the Centers for Disease Control and Prevention in 2000 were fluoroquinolone resistant (5). We have examined fluoroquinolone resistance and erythromycin resistance in *C. jejuni* isolated from patients seen at our institution since 1982. Previously we reported that fluoroquinolone resistance was not observed in isolates from 1982 to 1992 (6). In contrast to limited national data, we have observed a dramatic increase in fluoroquinolone resistance in *C. jejuni* since the mid-1990s.

The Study

The population we tested included patients treated by physicians within the University of Pennsylvania Health System, which encompasses several Philadelphia-area hospitals. Most isolates were from outpatients seen at the Hospital of the University of Pennsylvania or the Presbyterian Medical Center; both serve the University of Pennsylvania community and populations living in west Philadelphia. Stool samples were collected as part of the routine evaluation of patients with diarrheal illness and sent in Cary-Blair transport medium to the Clinical Microbiology Laboratory at the Hospital of the University of Pennsylvania for processing. *Campylobacter* organisms were isolated and identified to species by using published methods (7). Only *C. jejuni* subsp. *jejuni* were included in this study. Each isolate tested represents a single patient.

From 1995 through 2001, 404 patient isolates were obtained from routine stool cultures; 297 (73.5%) were available for susceptibility testing. The ratio of males to females was 1.15:1. The age distribution was nearly identical for both sexes (males: median 33 yrs, mean 35 yrs [range 1–86 yrs]; females: medium 33 yrs; mean 36 yrs [range 8–95 yrs]). Isolates were stored at –70°C and subcultured at least once before testing. Susceptibility to ciprofloxacin and erythromycin was determined with the E-test (AB Biodisk, Solna, Sweden) method. Organisms were tested on Mueller-Hinton blood agar medium and incubated at 37°C in microaerobic conditions. The breakpoints used for resistance were ≥4 µg/mL for ciprofloxacin and ≥8 µg/mL for erythromycin (5). Flagellin gene typing (Fla typing) was performed by using modified consensus primers, described by Wassenaar and Newell (8), and digested with *DdeI* as previously described (9).

As reported previously, fluoroquinolone-resistant *C. jejuni* were not detected among 142 patient isolates tested from 1982 to 1992 at our institution (6). Erythromycin resistance was 2.0% overall from 1982 to 1992. Two hundred and ninety-seven patient isolates were tested between 1995 and 2001 for susceptibility to ciprofloxacin and erythromycin. Resistance rates ranged from as low as 8.3% in 1996 to 40.5% in 2001 (Figure 1). In contrast, erythromycin resistance fluctuated between 0% and 5% during the same period; in 2001 erythromycin resistance was 3.5%. When all isolates tested during the study period are considered, 28.9% of isolates were resistant in the first calendar year quarter, 19.7% in the second quarter, 20% in the third quarter, and 19.2% in the fourth quarter. However, resistance isolates were more frequent beginning in October 2000 and extending through April 2001 (Figure 2).

Figure 3 shows the ciprofloxacin MIC distribution of isolates from 1995 to 2001. A clear bimodal distribution of MICs exists, with 96% of susceptible isolates with MICs ≤0.5 µg/mL; except for one isolate, all resistant isolates had MICs ≥32 µg/mL.

We used molecular typing by restriction fragment length polymorphism analysis of *Campylobacter flaA* to determine whether certain Fla types were associated with fluoroquinolone resistance. Twenty-nine different Fla types occurred

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[1]This study was presented in part at the 11th International Workshop on *Campylobacter, Helicobacter* and Related Organisms, September 1–5, 2001, Freiburg, Germany.

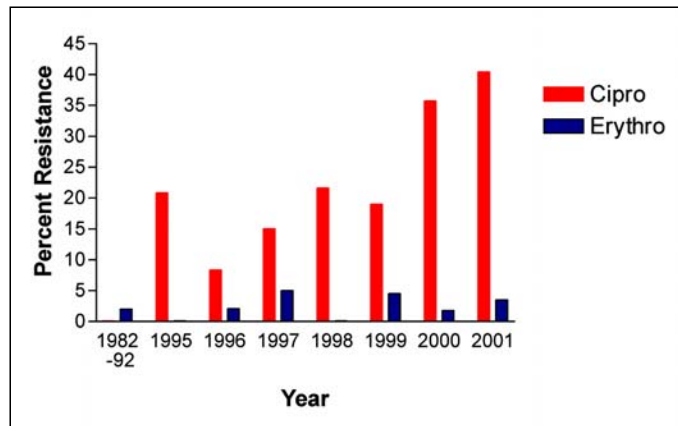

Figure 1. Trends in erythromycin and ciprofloxacin resistance in *Campylobacter jejuni*, Philadelphia, 1982–2001. Number of isolates tested: 1982–92 (n=142), 1995 (n=24), 1996 (n=48), 1997 (n=61), 1998 (n=37), 1999 (n=22), 2000 (n=48), and 2001 (n=47).

among the population of isolates. For strains where there were at least four isolates represented in the type (Fla types 1, 7, 9, 10, 13, 15, 16, 25, 33, 44, 48, 49, 53, 57, 80, 86), the proportion of resistant to susceptible isolates was no more than 0.25 and ranged from 0.07 to 0.25. None of the Fla types were specifically associated with fluoroquinolone resistance.

Conclusions

We have observed a dramatic increase in fluoroquinolone-resistant *C. jejuni* in patients treated within our health system from 1995 to 2001 with a resistance rate of 40.5% in 2001. In contrast, erythromycin-resistant *C. jejuni* has remained at a low rate (<5%) for nearly 20 years. Before 1992, fluoroquinolone-resistant *C. jejuni* had not been detected at our institution (6). Whether fluoroquinolone resistance emerged during 1993–1994 is unknown because isolates from that period were not available. Similarly, a survey of isolates from 19 U.S. counties in 1989 and 1990 did not find any fluoroquinolone-resistant *C. jejuni* (1). From 1997 through 2000, NARMS reported 13%, 13%, 18 %, and 14% fluoroquinolone-resistant *C. jejuni*, respectively (5). Erythromycin-resistant *C. jejuni* occurred in 8%, 3%, 2%, and 1% of isolates from 1997 to 2000 among isolates tested by NARMS; our data parallels these national data (5). The distribution of ciprofloxacin MICs among *C. jejuni* from our survey also parallels NARMS data between 1997 and 2000 (5). Fluoroquinolone-resistant isolates exhibited high-level resistance with MICs ≥32 µg/mL.

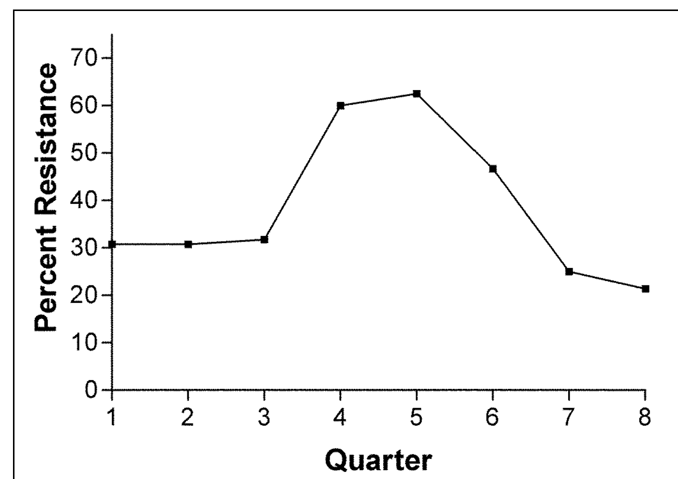

Figure 2. Fluoroquinolone-resistant *Campylobacter*, by quarter, 2000–2001. Number of isolates tested for each quarter: Q1: 13, Q2: 13, Q3: 22, Q4: 10, Q5: 16, Q6: 15, Q7: 12, Q8: 14.

The risk factors for acquiring fluoroquinolone-resistant *C. jejuni* in the United States have not been defined; however, foreign travel was identified by Smith and colleagues as an important risk factor (for 75% of fluoroquinolone-resistant *C. jejuni*) in Minnesota residents (4). Other, unidentified factors were important, however, since the rest of infections were domestically acquired. Use of a fluoroquinolone within the month before the collection of the stool sample was also identified as a potential risk factor (4). The increase in fluoroquinolone-resistant *C. jejuni* from 1996 through 1998 was temporally associated with the licensure of fluroquinolones for use in poultry in the United States (4). Several studies from European colleagues noted this temporal relationship between use of fluoroquinolones in animals and resistance among human isolates in the 1980s (3).

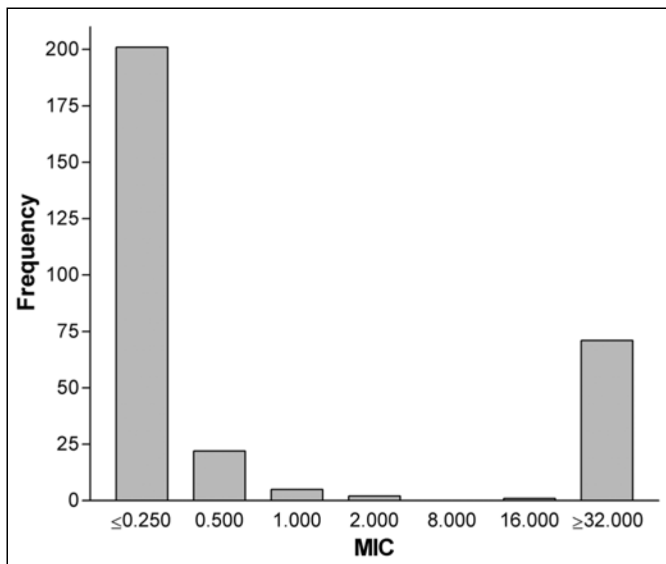

Figure 3. Distribution of ciprofloxacin MICs in *Campylobacter jejuni*, 1995–2001.

The reasons for such a dramatic increase in fluoroquinolone-resistant *C. jejuni* in our population are unknown. We examined the connection between seasonality and isolation of fluoroquinolone-resistant *C. jejuni.* We did observe increasing rates of resistance for several quarters during the last 2-year survey period. Whether this increase is indicative of foreign travel patterns by our patients is unknown. Future studies should focus on identifying the factors for acquisition of fluoroquinolone-resistant *C. jejuni* as well as the clinical implications of infection with such strains. Some evidence suggests that infection with fluoroquinolone-resistant *C. jejuni* results in prolonged illness. The duration of diarrhea among patients treated with a fluoroquinolone in the Minnesota study was sig-

nificantly longer if the patient had a fluoroquinolone-resistant infection (median 10 days) versus a fluoroquinolone-susceptible infection (median 7 days)(4). Based on national trends and our own local data, erythromycin continues to be the drug of choice for treating *Campylobacter* gastroenteritis.

Dr. Nachamkin is a professor of pathology and laboratory medicine and associate director of the Clinical Microbiology Laboratory, Hospital of the University of Pennsylvania. His main research interest is in the study of *Campylobacter* infections.

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New Variant of Varicella-Zoster Virus

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In 1998, a varicella-zoster virus glycoprotein E (gE) mutant virus (VZV-MSP) was isolated from a child with chickenpox. VZV-MSP, representing a second VZV serotype, was considered a rarity. We isolated another VZV-MSP-like virus from an elderly man with herpes zoster. These gE mutant viruses may have arisen through independent mutation or may represent a distinct VZV subpopulation that emerged more than 50 years ago.

In 1998, a VZV mutant virus (VZV-MSP) was discovered in Minneapolis-St. Paul, Minnesota; the virus had a missense mutation in the preponderant surface glycoprotein called gE (1,2). The mutation in VZV-MSP led to a lost B-cell epitope in the gE ectodomain. We define "mutant" as a virus with distinctive phenotypic characteristics associated with a nucleotide polymorphism and "variant" as a more inclusive term to include any virus with nucleotide polymorphisms whether or not the virus has a distinguishable associated phenotype. Many methods for differentiating VZV variants and their application to molecular epidemiologic studies have been described (3–8). However, no nucleotide polymorphisms, with the exception of the gE mutation in VZV-MSP, have conclusively been linked to distinguishable phenotypes.

Before 1995, a general assumption was that only one VZV serotype was found around the world (5,9). Similarly, the published sequence of the Dumas laboratory strain was generally accepted as the standard for all strains (10,11). Whether the VZV-MSP mutant virus exhibits increased fitness is not yet known. However, the mutant virus has a recognizable phenotype consisting of accelerated cell spread in both cell culture and the SCID-hu mouse (severe combined immunodeficient mouse with a human skin implant) model of VZV pathogenesis as well as a strikingly different pattern of egress, as documented by scanning electron microscopy (2). VZV-MSP was isolated from a child with leukemia who contracted chickenpox and was admitted to hospital for treatment with intravenous acyclovir. The discovery of a VZV gE variant virus was unexpected; one hypothesis is that the mutation occurred for the first time in the virus replicating in the index case and that the discovery was a serendipitous event unlikely to ever occur again.

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We describe the second case of a gE mutant virus found in North America. This discovery provides evidence that the gE mutant virus was unlikely to have occurred as a serendipitous chance mutation.

Case Report

In December 1999, a 75-year-old man from Vancouver, British Columbia, Canada, arrived at the hospital with severe zoster lesions on his face (in division V1 of the left trigeminal cranial nerve). Painful postherpetic neuralgia with headaches, associated with occasional blurring of vision and lasting for 6 months, developed. The patient was treated with famciclovir (500 mg three times a day for 7 days). A swab of the facial vesicles was obtained from the patient during acute zoster before famciclovir treatment.

Characteristic VZV cytopathogenic effect was seen after 9 days in cell culture. Results of immunofluorescence staining, performed with a commercial kit (Meridian Diagnostics, Cincinnati, OH) by using the anti-gE monoclonal antibody (MAb 3B3), were negative. Staining with monoclonal antibody conjugates for cytomegalovirus and human herpes simplex viruses was also negative. Electron microscopy indicated the presence of enveloped virus characteristic of herpesviruses.

Polymerase chain reaction amplification and subsequent DNA sequencing confirmed the virus genome to be VZV (hereafter referred to as VZV-BC). The VZV-BC strain was then analyzed in ORF68 (encodes gE and contains the 3B3 MAb epitope) and found to have an identical nucleotide sequence to VZV-MSP (GenBank accession no. AY005330). The VZV-BC and VZV-MSP strains differed from the prototype Dumas VZV strain (GenBank accession no. X04370) in ORF68 by a single G448A nucleotide mutation (D150N amino acid substitution) in the 3B3 epitope of gE (Table). VZV-MSP and VZV-BC were also identical in nucleotide sequence in ORF62 (IE62) and ORF37 (gH). In short, VZV-BC was closely related genetically, if not identical, to the VZV-MSP mutant virus.

Conclusions

VZV-MSP was the first community-acquired VZV strain shown to have a phenotype distinguishable from the traditional VZV phenotype. This case report documents the existence of a second isolate with the same gE genotype. VZV-MSP has been shown to have attributes consistent with increased virulence in the SCID-hu mouse model (2). Both VZV-MSP and VZV-BC are escape mutants (i.e., they have lost a B-cell epitope present in the prototypic Dumas virus from Holland, as well as other viruses from North America, Europe, and Asia, including the Oka varicella vaccine strain from Japan) (11). We have previously shown that the same gE epitope is involved in both complement-dependent neutralization (12) as well as antibody-dependent cellular cytotoxicity (13). Considering the cell culture and animal model data for VZV-MSP and the lost B-cell epitope resulting from the D150N gE mutation, this mutation should be monitored to determine whether it plays a role in

Table. Amino acid sequences of VZV strains Dumas, MSP and BC in the 3B3 monoclonal antibody epitope[a]

	Codon											
	150	151	152	153	154	155	156	157	158	159	160	161
Dumas	D	Q	R	Q	Y	G	D	V	F	K	G	D
VZV-MSP	N	Q	R	Q	Y	G	D	V	F	K	G	D
VZV-BC	N	Q	R	Q	Y	G	D	V	F	K	G	D

[a]VZV-BC, varicella-zoster virus found in British Columbia; VZV-MSP, varicella-zoster virus glycoprotein E mutant virus.

more severe cases of varicella or zoster or in breakthrough varicella in previously immunized children.

The origin of this variant may be the same gE mutation, occurring separately in two patients by chance. The argument favoring chance was raised after the discovery of the first gE mutant; however, we find this argument less compelling after the discovery of a second VZV strain with an identically mutated gE antigenic site, just 4 years later. Two other possibilities for its origin seem more plausible. The same gE mutation may have occurred from selective antibody pressure. In an article from the laboratory of the Nobel laureate Zinkernagel (14), the investigators showed that antibody escape variant viruses were more likely to arise in animals with deficient cellular immunity. The original VZV-MSP isolate was obtained from chickenpox in a child under treatment for leukemia, a condition known to be associated with depressed immunity. The second isolate was obtained from an elderly adult with herpes zoster, a condition that may be related to diminished immunity from aging. One of the antibody-escape glycoprotein mutant viruses described by the Zinkernagel laboratory had the identical aspartic acid to asparagine mutation seen in the two VZV-MSP glycoprotein variant viruses. However, mutant viruses probably would have been discovered in the immunocompromised population. The most likely scenario is that VZV-MSP is a previously unrecognized subpopulation of VZV that has been circulating for more than 50 years in circumscribed regions of northern United States and Canada. The timeline is based on the decade when the British Columbia patient likely first contracted chickenpox as a child. The gE sequencing analyses of 30 isolates by Shankar et al. (15) did not uncover a D150N gE mutation; therefore, larger studies need to be conducted to determine the prevalence of this mutant virus in North America.

We conclude that diagnosticians should be aware of the existence of VZV-MSP-like strains and the potential for false-negative testing results with single MAb-based antigen-detection kits for VZV or other herpesviruses. Further clinical observations are needed to fully assess the biological significance of the VZV gE mutant virus.

Dr. Tipples is a research scientist and head of the Viral Exanthemata Section at the National Microbiology Laboratory, Health Canada. His program is focused on the surveillance and diagnostics of viruses causing rash illnesses (measles, rubella, varicella-zoster virus, and human herpesvirus 6 and 7)

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Plasmodium ovale Malaria Acquired in Central Spain

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We describe a case of locally acquired *Plasmodium ovale* malaria in Spain. The patient was a Spanish woman who had never traveled out of Spain and had no other risk factors for malaria. Because patients with malaria may never have visited endemic areas, occasional transmission of malaria to European hosts is a diagnostic and clinical challenge.

In the first decades of the 20th century, malaria was a highly endemic disease in Spain. After the civil war (1936–1939), a large epidemic occurred; more than 293,000 cases and 1,278 deaths were reported (1). As a result, public health officials in Spain established strict control measures, and the disease was officially declared eradicated in 1964 (1). Malaria in Spain has been historically transmitted by *Anopheles atroparvus* and *An. labranchiae.* However, in recent entomologic surveys conducted in areas that were previously malarious, only *An. atroparvus* has been found in high densities similar to those observed during the years malaria was endemic (2,3).

Since the eradication of malaria in Europe, locally acquired malaria on the continent has usually been classified as "airport" or "odyssean" malaria (transmitted by infected mosquitoes transported by airplanes, ships, containers, luggage, buses, and the like) (4,5), and cases of malaria reported in Europe without identifiable risk factors have been classified as "cryptic" malaria (6). These cryptic cases may have occurred through local mosquito-borne transmission. In 1997, a case of malaria was diagnosed in a patient from southern Italy and identified as probable malaria transmitted by an autochthonous mosquito that fed on a gametogenic host; information on this case was shared with public health officials in Europe to reduce the risk of reintroducing malaria into the Mediterranean basin (7).

Because of its close proximity to Africa, Spain is one of the European countries most susceptible to the traffic of sub-Saharan migrant workers and the risk for transmission by mosquitoes migrating from other countries or indigenous anophelines infected by gametogenic hosts has increased substantially. We describe a case of malaria in a European woman who had never traveled out of Spain and had no other risk factors for malaria.

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The Study

In March 2001, a 75-year-old woman was admitted to the Hospital Príncipe de Asturias in Madrid with a history of intermittent fever for 1 week and no obvious infection. Intravenous treatment with ciprofloxacin was prescribed to treat provisionally diagnosed pyelonephritis. While in hospital, the patient had two episodes of high fever (39°C–40°C) separated by 48-hour intervals with hypoxemia and deterioration of her general condition. On day 7 of fever, the hematologist advised the physician of the presence of rings inside the patient's erythrocytes (parasitemia rate <1 %). A rapid antigen detection test (HRP2 detection; ICT Diagnostics, Amrad Corporation, Victor, Australia) was done; the test returned negative results for *Plasmodium falciparum* and *P. vivax*. The sample was later identified as *P. ovale* through microscopy and molecular studies at a reference malaria laboratory. Initial treatment with chloroquine followed by primaquine eliminated the infection successfully, and the patient recovered fully without complications.

P. ovale was confirmed by semi-nested multiplex polymerase chain reaction (PCR) (8). DNA isolation was carried out with Chelex (9) and the total DNA with the QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany); the sample was amplified, and a 499-bp fragment compatible with *P. ovale* was determined. PCR was repeated by using primers at two different temperatures, and the presence of *P. ovale* fragments was confirmed. The *P. ovale* fragments, initially embedded in the agarose gel, were then extracted with the help of the column for DNA purification. The rDNA was also amplified by using four specific primers (two forward and two reverse) at two different temperatures to obtain a large quantity of DNA for posterior sequencing. The product of amplification was sequenced in the ABISPRISM 377 XL DNA automatic sequencer (PE Applied Biosystems, Foster City, CA). Afterwards, sequences were sent to the GenBank database; in all the cases, 100% homology for the small subunit of the *P. ovale* rRNA gene was confirmed.

Epidemiologic Study

The patient had never traveled outside of Spain nor had any previous contact with people who had lived in or visited a country with endemic malaria. She had not received any packages from malaria-endemic areas. Because of obesity and instability from normotensive hydrocephalus, she had been confined to her home since January 2000, except for two visits to the hospital. She resides in an urban area close to two rivers (<1 km distance) and two international airports (Torrejón de Ardoz [4 km distance] and Barajas [18 km distance]). The city in which the patient lives (Alcalá de Henares) is very close to the Spanish capital, Madrid (30 km), an area with a meso-Mediterranean climate characterized by 400–500 mm of annual rainfall and average temperatures of 6°C in winter and 22°C–24°C in summer. The city's geographic conditions are semiarid with 3–4 months of dry seasons. Alcalá has 180,060 inhabitants, including 12,711 (7%) foreign residents; 1,121 (0.6%) of the residents are Africans from malaria-endemic

countries located mostly in the western and central regions of Africa.

We investigated the patient's medical history for other risk factors for malaria. The patient's uterus, ovaries, gall bladder, and appendix were removed >30 years ago, and she had never received any blood transfusions or blood derivates. Other risk factors such as needle-sharing, malariotherapy, or organ transplants were discarded. The possibility of iatrogenic transmissions during previous hospital admissions (the last visit was 3 weeks before onset of symptoms) was also investigated and ruled out.

In the city in which the patient resides, the incidence of malaria in 1999 and 2000 was 1.2 and 3.7 cases per 100,000 inhabitants, respectively, but no cases were produced by *P. ovale*. In contrast, in the region surrounding Madrid, which includes two international airports, the incidence of imported malaria in 1999 and 2000 was, 2.7 and 3.4 cases per 100,000 inhabitants, respectively, (139 and 171 cases in total); *P. ovale* was the species implicated in 2.1% and 5.3% of these regional cases, respectively. Health care in Spain is free for foreign residents, but they must be inscribed in the local census bureau, and many illegal foreign migrant or temporary workers are likely not covered by any health insurance.

Conclusions

This case is the first locally acquired *P. ovale* infection connected with Europe. The infection may have been transmitted by an odyssean vector. The patient lives 4 and 18 km away from international airports, within the radius of other previously reported airport malaria cases (4,10). The parasite may also have been transmitted by a local mosquito (introduced malaria) (5). In Spain, a possible vector for local infection is *An. atroparvus*, since this species has shown receptivity to *P. vivax* (11) and possibly could be receptive to *P. ovale* as well. Surprisingly, the illness began when cold temperatures prevailed. Thus, the disease could have been a relapse from hepatic hypnozoite or a primoinfection produced by the bite of an inhouse hibernating infected *Anopheles* spp. female (12). Whatever the mechanism, the diagnosis was complicated by the fact that the disease occurred during the winter.

Spain hosts a growing number of migrant workers from west and central Africa traveling through the Gibraltar Strait. Tourism and international flights to and from tropical countries have multiplied in recent years (13). All these factors can account for occasional or epidemic reintroduction of malaria into the country.

Although some studies indicate that indigenous mosquitoes such as *An. labranquiae* and *An. atroparvus* are not susceptible to the afrotropical *P. falciparum* strains (2,11), these species of anophelines are probably fully susceptible to infection by *P. vivax* and *P. ovale* strains imported from Africa. In addition, Asian or American *P. falciparum* strains may also be imported.

In Europe, malaria transmission can also occur in urban settings given the appropriate conditions. Malaria should be considered in patients with a fever of unknown origin, even if they have never traveled to malaria-endemic areas. Increased attention should be given to persons who work or live close to international airports or in areas with high population of new foreign residents from malarious areas. Hospital laboratories should be ready to detect malaria parasitemia on a 24-hour basis (thick films or antigen detection if microscopy expert is unavailable) on a physician's request. Likewise, PCR techniques should be used to detect retrospectively low parasitemias and confirm the species diagnosis. We suggest that epidemiologic and clinical attention should be given to travelers and newly arrived foreign residents from malaria-endemic countries to prevent secondary cases (14). Simple, easy access to health care for recently arrived migrant workers should be implemented to assess risk factors and screen for malaria if necessary.

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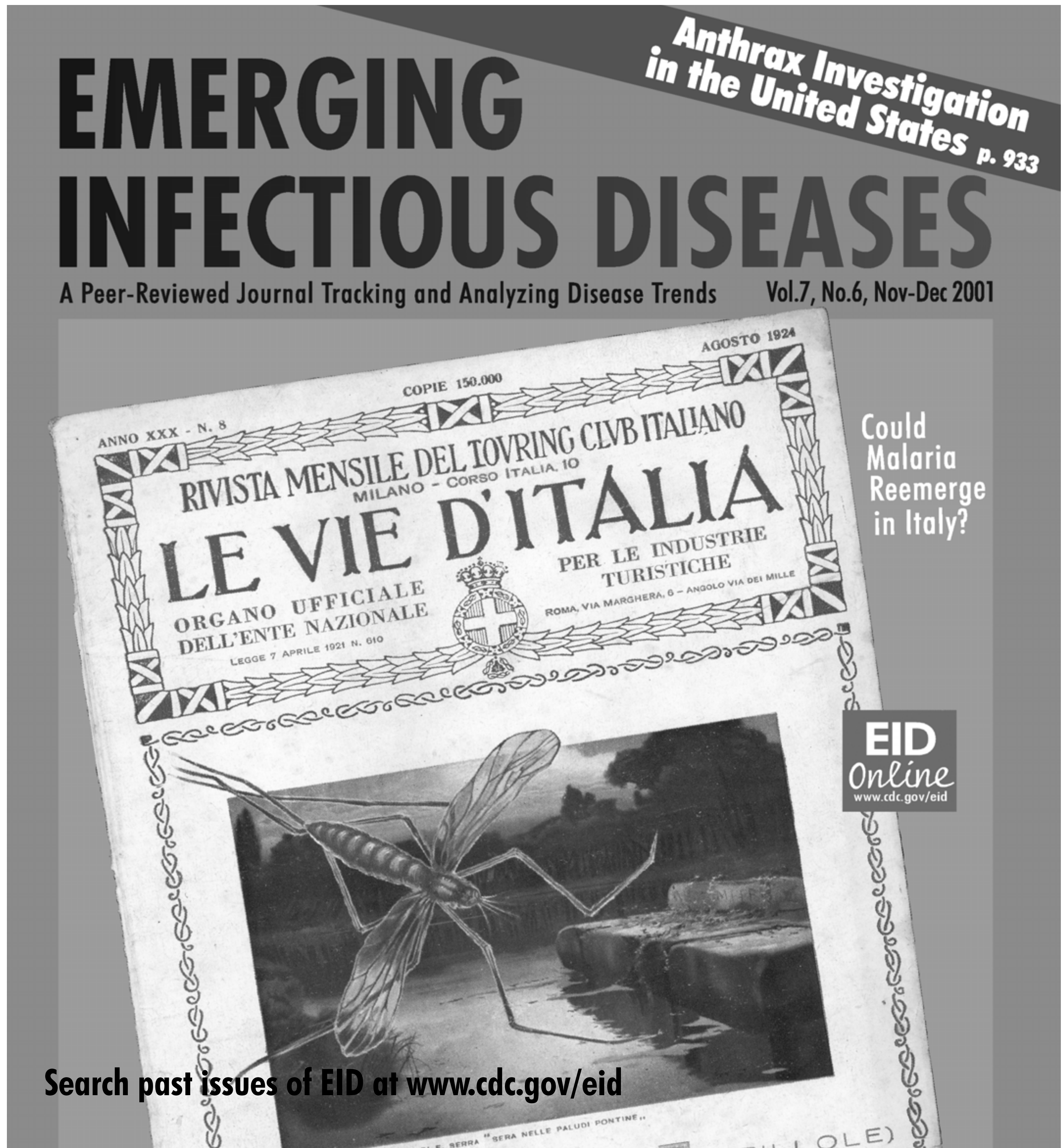

Puumala hantavirus Infection in Humans and in the Reservoir Host, Ardennes Region, France

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We compared the occurrence of nephropathia epidemica cases, over a multi-annual population cycle, in northeastern France with the hantavirus serology for bank voles captured in the same area. We discuss hypotheses to explain the pattern of infection in both humans and rodents and their synchrony.

In Eurasia, hantaviruses (family *Bunyaviridae*) are the etiologic agents of hemorrhagic fever with renal syndrome (HFRS) in humans (1). In France, the HFRS-endemic area is the northeastern quarter of the country (2–4). The Ardennes massif at the Belgian border hosted 244 recorded human cases during 1991–1999 and accounted for two thirds of the total number of French cases during the 1996–1999 regional bank vole (*Clethrionomys glareolus*) demographic cycle. Historically, 40% of all recorded French cases occur in this region. Human cases of nephropathia epidemica (NE), a milder form of HFRS caused by *Puumala virus* (PUUV), are routinely recorded at the Centre Hospitalier Régional (CHR) of Charleville, (Ardennes) France, which has the largest number of clinical cases in the country. Epidemic outbreaks of acute infection have been observed every third year since 1991. We hypothesize that the risk of human infection results from an increase in the mass shedding of virus, after a population increase of infected voles over a threshold density. In this case, the prevalence of infection in rodents may rise some time before the outbreak occurs in humans.

To test this hypothesis, we set up a surveillance protocol to investigate if increased densities of bank vole populations would amplify anti-PUUV antibody prevalence in the reservoir, reflecting an increased risk for infection by human beings. We determined the prevalence of anti-hantavirus antibodies in a population of bank voles, the rodent reservoir (5), within the disease-endemic area. During the 3 years before the last peak of disease (1997–1999), we monitored bank vole populations by trapping and screening antibodies. Animals were trapped in the Elan and Hazelles forests near the city of Charleville by using 100-m trap lines, each containing 34 non-baited small rodent INRA box traps (6) in the spring, summer, and autumn of 1997, 1998 (plus one session in December 1998), and 1999. The forests were divided into equal sectors, and 12 trap lines by forest (13 in 1997) were set in randomly chosen sectors for each capture session. Traps were checked daily for 3 successive days, and captured rodents were removed for further virologic studies. If lines were reset in the same sector on successive occasions, they were moved to reduce bias caused by the removal of animals trapped in the previous session.

Serum samples of blood from the trapped animals were withdrawn by cardiac puncture and evaluated by enzyme-linked immunosorbent assay (ELISA) on plaques directly coated with antigen from cells infected with PUUV or controls, lysed in triton-borate buffer, and then sonicated. Antibody uptake was estimated with peroxidase-tagged anti-mouse immunoglobulin (Ig) G, which cross-reacts with bank vole Ig (not shown). Positive serum samples were confirmed by immunofluorescence on PUUV or *Haantan* virus-infected Vero E6 cells at serial doubling dilutions. We did not use direct detection of the virus by reverse transcriptase polymerase chain reaction (RT-PCR). Therefore, a discrepancy between our estimates of infected voles and the true number of potentially infectious animals may have occurred but should not affect the temporal trends over a 3-month intervals (7,8).

During the study, we observed a fourfold increase in the density index of the monitored vole population, with seasonal fluctuations (highest in September and lowest in spring). A total of 550 animals were trapped during 25,092 trap nights for an overall trap success rate of 2.19% (3.25% in Elan, with 408 captures and 1.13% in Hazelles, with 142 captures). Five species of rodents and two species of insectivores were captured. Overall, *C. glareolus* was most commonly collected (49.8%); however, the proportion of the different species varied greatly between the two forests: bank voles accounted for 57.6% of captures in Elan but only for 27.5% of captures in Hazelles (chi-square=13.65, $p = 2.10^{-4}$), where they were overtaken in frequency by *Apodemus flavicollis* (29.6%). Twenty-nine rodents were seropositive for hantaviruses; 25 were bank voles (23 in Elan and 2 in Hazelles) and 4 were *A. sylvaticus* (3 in Elan and 1 in Hazelles). Seropositive wood mice were detected during the peak of prevalence in bank voles, which suggests a spillover infection similar to that seen in humans. We focused on bank voles in Elan because the number of infected voles from Hazelles is too small to allow statistical analysis. Among the 235 bank voles captured, 113 were male, 119 were female, and 3 were undetermined. The overall seroprevalence was 9.8% (23 seropositive voles), with 13 males (prevalence of 11.5%), 9 females (7.6%), and 1 undetermined. Prevalence did not differ between sexes (chi-square=0.64, p=0.42). Hantavirus antibody prevalence reached a maximum of 29% (4 of 14 bank voles tested) during the spring of 1999 from 8.9% (5/56) in the previous fall. Prevalence then fell to 11% (4/36) the next summer and remained at this level (7/76)

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until the population peak in September 1999. One might hypothesize that the amount of virus available for human contamination reached its highest level between September 1998 and September 1999.

We observed an irregular distribution of seropositive animals among the captures from the Elan forest (Figure 1); many trap lines did not have positive voles from a large number of captures, whereas others had higher rates (up to 3 of 3 captured voles). No clear correlation exists between host density (as estimated by capture frequency) and seropositivity, but seropositive animals were more often taken on northerly facing trap lines (c^2=12.68, p=4.10^{-4}) than southerly facing ones (c^2=0.86, p=0.35). This difference is probably due to the higher humidity of northerly facing slopes, which are less often exposed to the sun. Verhagen et al. (9) have reported that the probability that a bank vole will be infected increases with the humidity of its territory. The lower number of observations from the Hazelles forest also showed a variation in population density and antibody prevalence in synchrony with the Elan voles.

At least 40 human cases of NE (range 40–74) were recorded at the Charleville CHR in 1993, 1996, and 1999, whereas no more than 14 NE cases were seen in the intervening years (Figure 2). If the sampled rodents are representative of the whole reservoir population to which humans are exposed, our findings suggest a synchrony of infection rates in humans and reservoir rodents over the 3-year epidemiologic cycle. In accordance with previous records, the greatest number of HFRS cases were registered at Charleville CHR during the periods of highest prevalence in the reservoir host (1993, 1996, and 1999). Provided that the data from this preliminary study are accurate, the temporal correlation between infection rates in human victims and in the reservoir host (Spearman's rank correlation, z=2.55, p=0.01 p= 0.86) strongly suggests a common process of infection. The assumption that the vole demographic population peak precedes the epidemic outbreak in humans is not supported by our data. If our study is representative of the actual situation, our results suggest that the maximum infection rate is reached simultaneously in both the human and reservoir hosts. These results could be explained if the proportion of newly infected voles is more important than the total number of infected voles. In fact, the amount of virus shed during the first month of infection is far higher than during the consecutive chronic phase (10). In this case, the increased mass shedding of virus would not necessitate a very high prevalence. To attain the observed high proportion of infected voles for less than 1 month, even with a stable prevalence, the transmission between voles must be rapid in the increasing population.

We hypothesize that the mechanism of virus circulation is related to the social structure of the reservoir: bank voles are territorial and avoid encounters with conspecifics during the breeding season but share nests during winter. Direct transmission seems difficult during the reproductive season. We have previously examined a possible role of the environment in the survival of the virus outside its host to explain its observed patchy distribution (11). Human contamination occurs mainly by this indirect route (12). The bank vole social component, in combination with an indirect transmission route, can explain the rapid spread of infection through a population of increased density. The voles may become infected through the sniffing of contaminated excreta marks, even though these animals avoid direct encounters. This activity could result in a high proportion of newly contaminated voles. A change in the population dynamics of the bank vole reservoir, leading to a low incidence of newly infected individuals as seen in the more stable populations, could explain the transition from an epidemic to a sporadic pattern of HFRS in regions of France south of the Ardennes (2). This hypothesis will be tested in ongoing epidemiologic studies.

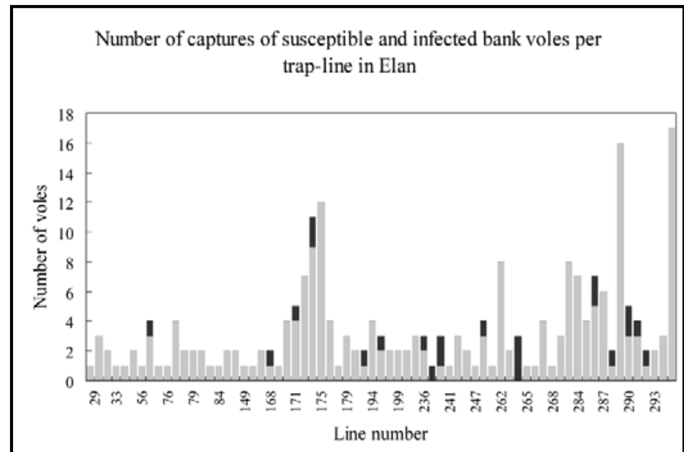

Figure 1. Number of captured bank voles (susceptible in light grey, seropositive in black) per trap line in Elan.

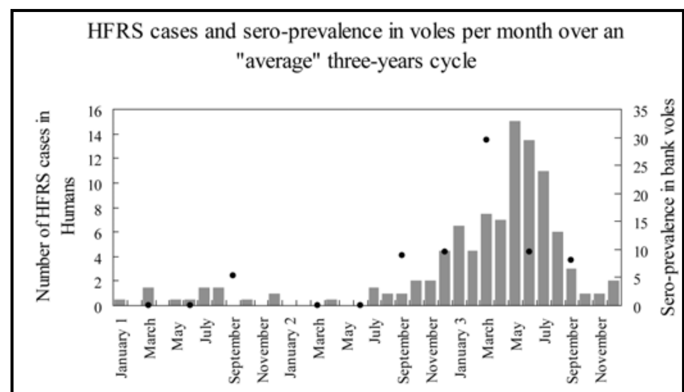

Figure 2. Temporal correspondence of reservoir contamination and of human cases of nephropathia epidemica over a typical 3-year cycle. Grey bars: the number of HFRS cases in humans per month for the Ardennes region (France) from 1991 to 1996; black points: observed hantavirus antibody prevalence in bank voles by trapping session in Elan forest over the same period. Right scale: percentage of seropositive voles in the trapped sample.

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Mr. Sauvage is a doctoral student under the supervision of Dominique Pontier. His main interests are ecological field studies on the epidemiology of hantaviruses in France and modeling approaches to these studies.

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Capsule Switching among C:2b:P1.2,5 Meningococcal Epidemic Strains after Mass Immunization Campaign, Spain

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A mass immunization campaign for 18-month to 19-year-olds was undertaken in Spain in 1996–1997 because of an epidemic of serogroup C meningococcal disease associated with a C:2b:P1.2,5 strain belonging to the A4 lineage. Surveillance for the "capsule-switching" phenomenon producing B:2b:P1.2,5 isolates was undertaken. Of 2,975 meningococci characterized, B:2b:P1.2,5 and B:2b:P1.2 antigenic combinations were found in 18 isolates; 15 meningococci were defined as serogroup B belonging to the A4 lineage.

In the early 1990s, an increasing number of serogroup C meningococcal strains were observed in Spain (1). Besides a change in the predominant serogroup, an increase in the incidence of the meningococcal disease associated with a new variant of serogroup C (2) was detected. These strains were characterized as C:2b:P1.2,5. Their frequency in serogroup C meningococci in Spain increased from 4.6% in 1993 to 65% in 1996 (2). Meningococcal strains characterized as C:2b:P1.2,5 have been described in other countries (3), but they have not been associated with a similar epidemiologic change. However, C:2a:P1.2 isolates belonging to the ET15 lineage have been responsible for epidemic waves in the Czech Republic and Canada (4,5).

As a result of the increase in such isolates in Spain, a mass immunization campaign focused at 18-month to 19-year-olds was conducted with the polysaccharide A+C vaccine in most of the country in 1996–1997 (6). Three years later, a new C conjugate vaccine was licensed in Spain. This vaccine was routinely introduced in autumn 2000 because an increase in the incidence of serogroup C cases was again detected.

By contrast, B:2b meningococci, which were frequently isolated during a previous epidemic period in Spain (7), represented 1.9% of the serogroup B strains characterized in our laboratory from 1990 to 1992 (8); none of them showed P1.2,5 serosubtype antigenic combinations. From 1995 to autumn 2000 we characterized 18 meningococcal strains as B:2b (14 as B:2b:P1.2,5 and 4 as B:2b:P1.2 isolates). Recombinant strains expressing serogroup B or C have been previously described as result of capsule-switching genetic mechanism (9,10). The aim of our study was to characterize those new B:2b meningococci variants. We used molecular typing methods (pulsed-field gel electrophoresis [PFGE] and multilocus sequence typing [MLST]) to determine the relationships among B:2b with the parental C:2b:P1.2,5 epidemic strain before and after the immunization campaign with the A+C polysaccharide vaccine.

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The Study

The Spanish Reference Laboratory for Meningococci routinely receives meningococci isolated from sterile sites for serogrouping, serotyping, and serosubtyping. From January 1995 to November 2000 (just before the new C conjugate vaccine was routinely introduced), the laboratory received 2,975 meningococcal strains to be characterized by serotyping and serosubtyping with monoclonal antibodies (8). The B:2b:P1.2,5 and B:2b:P1.2 antigenic combinations were found in 18 isolates (Table 1). All these strains were suspected of belonging to the A4 lineage and were fully characterized by PFGE and MLST as described previously (2,11); results were compared with those obtained among the C:2b:P1.2,5 epidemic strains. Two additional strains characterized as B:4:P1.2,5 were also included to determine if these antigenic combinations might be caused by similar genetics events.

Conclusions

Fifteen (83.3%) meningococci showed sequence types identified as representative of the A4 clonal lineage; this lineage also represents the genotype of the C:2b:P1.2,5 epidemic strain. The proportions of isolates belonging to the A4 clonal lineage were 75% and 85%, respectively, in both B:2b:P1.2 and B:2b:P1.2,5 strains. Seven of these 15 meningococci characterized as serogroup B belonging to the A4 lineage were isolated from patients who had never been immunized with the A+C polysaccharide vaccine. Three group B strains that were suspected by antigenic characterization of belonging to the A4 complex showed nonrelated sequence types (Table 1).

In a different study (data not shown), most of the C:2b:P1.2,5 epidemic strains grouped in two closely related pattern profiles by PFGE: PT7 and PT8. Table 1 shows the PFGE pattern profiles of the B:2b:P1.2,5 strains. Most of these isolates also showed the PT7 or the PT8 profile. Some strains showed minor pattern profiles already present among C:2b:P1.2,5 isolates (PT1, PT4, and PT38, all of them closely related to PT7 and PT8). Those strains showing PFGE pattern profiles that we did not find among the C:2b:P1.2,5 epidemic strain belonged to lineages different than A4 (Table 1). On the other hand, the B:4:P1.2,5 strains showed the same sequence type, ST33, associated with ET5.

The frequency of the C:2b:P1.2,5 and B:2b:P1.2,5 meningococci of the A4 lineage is shown in Table 2. A slight

Table 1. Distribution and characteristics of B:2b meningococcal strains with different antigenic combinations, Spain

Year	Identification no.	Antigenic expression	Pulse type	Clonal lineage by MLST[a]
1995	9813	B:2b:P1.2,5	NR[b]	ST1380[c]
	9976	B:2b:P1.2,5	PT7	ST8 (A4)
	10034	B:2b:P1.2,5	NR	ST1489[c]
1996	10317	B:2b:P1.2,5	PT7	ST8 (A4)
1997	11261	B:2b:P1.2	PT1	ST8 (A4)
	11327	B:2b:P1.2,5	PT7	ST8 (A4)
1998	12344	B:2b:P1.2,5	PT7	ST8 (A4)
	12366	B:2b:P1.2,5	PT7	ST8 (A4)
1999	12531	B:2b:P1.2	PT8	ST8 (A4)
	12644	B:2b:P1.2,5	PT8	ST8 (A4)
	12647	B:2b:P1.2,5	PT8	ST8 (A4)
	12792	B:2b:P1.2,5	PT8	ST8 (A4)
	12367	B:2b:P1.2,5	PT8	ST8 (A4)
2000	13602	B:2b:P1.2	PT38	ST8 (A4)
	13818	B:2b:P1.2,5	PT7	ST8 (A4)
	13872	B:2b:P1.2,5	PT4	ST66 (A4)
	13903	B:2b:P1.2	NR	ST162[c]
	14078	B:2b:P1.2,5	PT4	ST66 (A4)

[a]MLST, multilocus sequence typing.
[b]NR, pulse types not related to those found in C:2b:P1.2,5 strains.
[c]Clonal lineage not defined.

increase of that group B isolates was found after the immunization campaign.

Recombinant strains expressing serogroup B or C have been previously described as resulting from a capsule-switching genetic mechanism (9,10). A similar event with W135 isolates has been recently described (12). Thus, all the serogroups should be capable of changing to any other. However, the relevance of this phenomenon has not been fully described. In our surveillance analysis, the group B strains of the A4 lineage appeared before the vaccination campaigns; this finding differs from results of an analysis conducted in Canada after a similar surveillance (10). Our findings show that those genetic variants are being produced in the meningococcal population at random and that a variant's appearance is not necessarily related to mass immunization campaigns. In fact, these group B meningococci belonging to the A4 lineage were also isolated in some of the regions that used the vaccine on a small scale (13).

However, the increased number of these B:2b:P1.2,5 strains from the A4 lineage during the study period might indicate a positive selection caused by mass immunization campaigns during 1996 and 1997 (Table 2). Seven (50%) of these strains were isolated from patients who did not receive A+C vaccine, indicating that the individual immune status should not be a critical factor for developing meningococcal disease with these serogroup B strains of the A4 lineage rather than other clonal lineages. Nasopharingeal competition might be an important factor in the spread of these group B strains, as has been suggested to explain the spread of serogroup B meningococci belonging to the ET15 lineage (10). Theoretically, however, the C:2b strains of the A4 lineage and the B:2b meningococci also belonging to the A4 lineage should have a very similar genetic background with the exception of a locus in the capsular operon (9). In fact, both types of strains showed not only the same or similar sequence type by MLST but also a very similar genetic profile by PFGE (data not shown). Thus, a similar epidemic potential in these two capsular variants might be expected. Nevertheless, those strains with a group C polysaccharide capsule maintained a major epidemic even after a mass immunization campaign (Table 2) and even when these group C strains were not common in the carrier population (14).

Whether a similar observation can be made for the serogroup B strains belonging to the A4 clonal lineage is not clear; the small increase in these serogroup B strains does not appear to be linked with increased epidemic potential. Thus, the capsular polysaccharide appears to be the only differing factor between these two types of meningococci. Once again, the high number of serogroup B strains in asymptomatic carrier population (14) associated with a natural immunity, might partially explain the different epidemic potential of the two genotypes. However, the nature of the group C polysaccharide alone does not explain why C:2b:P1.2,5 strains were responsible for an important epidemic wave in Spain in 1996–1997. Some other factors, such as the amount of polysaccharide, might explain differences in virulence (15).

Two meningococci characterized as B:4:P1.2,5 were included to analyze if some other antigenic combinations might appear as result of different genetic events. This possibility was not confirmed in our study but should be more accurately analyzed in the future.

In 2000, a new increase in cases of group C meningococcal disease was detected in some regions of Spain (6). Because of these data, Spanish health authorities made the decision to include a new group C conjugate vaccine in the routine infant immunization schedule beginning in autumn 2000. How the two different vaccines against group C meningococci

Table 2. Frequency of the B:2b:P1.2,5 and C:2b:P1.2,5 meningococcal strains characterized by the laboratory, 1995–2000

Year	No. of strains received by the laboratory	No. of C:2b: P1.2,5 (%)	No. of B:2b: P1.2,5 (%)
1995	254	56 (22%)	1 (0.4%)
1996	380	176 (46.3%)	1 (0.26%)
1997	687	313 (45.5%)	2 (0.3%)
1998	554	149 (26.9%)	2 (0.36%)
1999	598	144 (24.1%)	5 (0.84%)
2000	502	97 (19.3%)	4 (0.8%)

(polysaccharide and conjugate) influence the selection of serogroup B belonging to the A4 lineage strains in Spain that have had a capsular-switching event would be an interesting future topic of research.

Acknowledgments

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Dr Belén Alcalá is a postdoctoral fellow in the Spanish Reference Laboratory for Meningococci. Her research has been mainly focused on the analysis of the genetic relationships among the meningococcal strains isolated in Spain during an outbreak of serogroup C cases during 1996 and 1997. Her professional interests also include the study of mechanisms for genetic regulation of antigens in *Neisseria meningitidis*.

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Human Pathogens in Body and Head Lice

Pierre-Edouard Fournier,*
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Patrick J. Kelly,§ and Didier Raoult*

Using polymerase chain reaction and sequencing, we investigated the prevalence of *Rickettsia prowazekii*, *Bartonella quintana,* and *Borrelia recurrentis* in 841 body lice collected from various countries. We detected *R. prowazekii* in body lice from Burundi in 1997 and in lice from Burundi and Rwanda in 2001; *B. quintana* infections of body lice were widespread. We did not detect *B. recurrentis* in any lice.

The body louse, *Pediculus humanus corporis*, is the vector of three human pathogens: *Rickettsia prowazekii*, the agent of epidemic typhus; *Borrelia recurrentis*, the agent of relapsing fever; and *Bartonella quintana*, the agent of trench fever, bacillary angiomatosis, endocarditis, chronic bacteremia, and chronic lymphadenopathy (1). Louse-borne diseases can be associated with high incidence of disease and death, especially epidemic typhus and relapsing fever, which can be fatal in up to 40% of patients (2). The diseases are mostly prevalent in people living in poverty and overcrowded conditions, for example, homeless people and those involved in war situations (2).

Epidemic typhus, trench fever, and relapsing fever have been the subject of many studies, most of which were conducted between World War I and the 1960s. However, medical interest in the diseases and lice waned for almost 30 years. Since 1995 louse-borne diseases have had a dramatic resurgence, and trench fever has been diagnosed in many countries including the USA (3), Peru (4), France (5), Russia (6), and Burundi (7). In 1997 the largest outbreak of epidemic typhus since World War II occurred in Burundi among refugees displaced by civil war (7). A small outbreak also occurred in Russia (8), and evidence of *R. prowazekii* infection in Algeria was provided (9).

At the Unité des Rickettsies, we developed a polymerase chain reaction (PCR) assay to survey for human pathogens transmitted by the parasites; the assay can detect as few as 1–20 copies of the DNA of *R. prowazekii*, *B. quintana,* and *Borrelia recurrentis* in body lice (10). In 1995, we found *R. prowazekii*–positive lice in inmates of a Burundi jail (11), which was the source of a major outbreak of epidemic typhus in the country in 1996 (12). In 1997, we investigated an outbreak of pediculosis in refugee camps in Burundi. We identified *R. prowazekii* and *B. recurrentis* in body lice and epidemic typhus and trench fever in refugees (7,10). From April 1997 to December 1998, after our reports, a new strategy was designed to control typhus and trench fever. Health workers treated any patient with fever >38.5°C with a single dose of doxycycline (200 mg), a drug highly effective in the treatment of typhus (7). The program proved extremely successful, and in a follow-up in 1998 (10) we did not detect *R. prowazekii* in body lice collected in refugee camps in the country (Table 1).

Since 1998, we have continued our efforts and have collected 841 body lice obtained by medical staff from our laboratory or local investigators in Burundi, Rwanda, France, Tunisia, Algeria, Russia, Peru, China, Thailand, Australia, Zimbabwe, and the Netherlands (Table 1). In Burundi, lice were collected during the outbreak of epidemic typhus and on three occasions (1998, 2000, and 2001) after the outbreak had been controlled. Lice found on any part of the body, except the head and pubis, were regarded as body lice. The lice were transported to France in sealed, preservative-free, plastic tubes at room temperature. Delays between collection and analysis ranged from 1 day to 6 months. As negative controls, we used specific pathogen-free laboratory-raised body lice (*Pediculus humanus corporis* strain Orlando). To prevent contamination problems, as positive controls we used DNA from *R. rickettsii* R (ATCC VR-891), *Bartonella elizabethae* F9251 (ATCC 49927), and *Borrelia burgdorferi* B31 (ATCC 35210), which would react with the primer pairs we used in our PCRs but give sequences distinct from the organisms under investigation. To prevent false-positive reactions from surface contaminants, each louse was immersed for 5 min in a solution of 70% ethanol–0.2% iodine before DNA extraction and then washed for 5 min in sterile distilled water. After each louse was crushed individually in a sterile Eppendorf tube with the tip of a sterile pipette, DNA was extracted by using the QIAamp Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. This kit was also used to extract DNA from the organisms cultivated in our laboratory under standard conditions to be used as positive controls. The effectiveness of the DNA extraction procedure and the absence of PCR inhibitors were determined by PCR with broad-range 18S rDNA-derived primers (10).

To detect louse-transmitted pathogens, we used each of the genus-specific primer pairs described in Table 2 in a separate assay. A total of 2.5 mL of the extracted DNA was used for DNA amplification as previously described (10). PCRs were carried out in a Peltier Thermal Cycler PTC-200 (MJ Research, Inc., Watertown, MA). PCR products were resolved by electrophoresis in 1% agarose gels. All lice yielded positive PCR products when amplified with the 18S rRNA-derived primers, demonstrating the absence of PCR inhibitors. Negative controls always failed to yield detectable PCR products, whereas positive controls always gave expected PCR products. PCR amplicons were purified by using the QIAquick Spin PCR purification kit (Qiagen) and sequenced using the dRhodamine Terminator cycle-sequencing ready reaction kit (PE Applied Biosystems, Les Ulis, France), according to the

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Table 1. Prevalences of infections in body lice collected in various areas of the world

Country	Source, yr	Reference[b]	No.	Detection[a] of	
				Rickettsia prowazekii (no., %)	*Bartonella quintana* (no., %)
Body lice					
France	Homeless in Marseille, 1998–2001	PS[c]	324	0	32 (9.9%)
France	Homeless shelter in Marseille, 2000	(13)	161	0	42 (26.1%)
France	Isolated homeless in Marseille, 1998	(10)	75	0	3 (4.0%)
The Netherlands	Homeless in Utrecht, 2001	PS	25	0	9 (36.0%)
Russia	Homeless in Moscow, 1998	(10)	268	0	33 (12.3%)
Tunisia	Homeless in Sousse, 2000	PS	3	0	0
Algeria	Homeless in Batna, 2001	PS	33	0	0
Congo	Refugee camp, 1998	(10)	7	0	0
Burundi	During typhus outbreak				
	Jail, 1997	(10)	10	2 (20%)	0
	Refugee camp, 1997	(10)	63	22 (35%)	6 (9.5%)
	After typhus outbreak				
	Refugee camp, 1998	(10)	91	0	13 (14.3%)
	Refugee camp, 1998	PS	38	0	8 (21.0%)
	Refugee camp, 2000	PS	111	0	100 (90%)
	Refugee camp, 2001	PS	33	7 (21%)	31 (93.9%)
Rwanda	Jail, 2001	PS	262	19 (7%)	6 (2.3%)
Zimbabwe	Homeless in Harare, 1998	(10)	12	0	2 (16.7%)
Australia	Homeless in , 2001	PS	2	0	0
Peru	Andean rural population	(10)	73	0	1 (1.4%)
Peru	Andean rural population	PS	10	0	0
Head lice					
France	Schoolchildren	PS	20	0	0
Portugal	Schoolchildren	PS	20	0	0
Russia	Schoolchildren	PS	10	0	0
Algeria	Schoolchildren	PS	18	0	0
Burundi	Schoolchildren	PS	20	0	0
China	Schoolchildren	PS	23	0	0
Thailand	Schoolchildren	PS	29	0	0
Australia	Schoolchildren	PS	3	0	0

[a]*Borrelia recurrentis* could not be detected in any of the tested lice.
[b]Data previously reported in the indicated reference.
[c]PS, present study.

manufacturer's recommendations. Sequences obtained were compared with those in the GenBank DNA database by using the program BLAST (14).

The sequences of the DNA amplicons we obtained were identical to those of *R. prowazekii* and *B. quintana* in GenBank. We detected *R. prowazekii* in body lice collected in Burundi in 2001 but not in those collected in 1998 and 2000, although they were positive for *B. quintana*. *R. prowazekii* was also detected in 7% of lice collected in Rwanda. We found *B. quintana* in body lice collected in France, the Netherlands, Russia, Burundi, Rwanda, Zimbabwe, and Peru. No PCR products were obtained for any of the lice when primer pair Bf1-Br1 was used, indicating lack of infections with *Borrelia recurrentis*.

Our PCR may greatly facilitate the study of lice and louse-borne diseases as it can be used to survey lice for these organisms, detect infected patients, estimate the risk for outbreaks, follow the progress of epidemics, and justify the implementa-

Table 2. Oligonucleotide primers used for PCR amplification and sequencing[a]

Primer (reference)	Nucleotide sequence	Organism or sequence used	Size of expected PCR product (bp)
CS-877 (10)	GGG GGC CTG CTC ACG GCG G	*Rickettsia* species	396
CS-1273 (10)	ATT GCA AAA AGT ACA GTG AAC A	*Rickettsia* species	
QHVE1 (10)	TTC AGA TGA TGA TCC CAA GC	*Bartonella* species	608
QHVE3 (10)	AAC ATG TCT GAA TAT ATC TTC	*Bartonella* species	
Bf1 (10)	GCT GGC AGT GCG TCT TAA GC	*Borrelia* species	1,356
Br1 (10)	GCT TCG GGT ATC CTC AAC TC	*Borrelia* species	
18saidg (10)	TCT GGT TGA TCC TGC CAG TA	Arthropods	1,526
18sbi (10)	GAG TCT CGT TCG TTA TCG GA	Arthropods	

[a]PCR, polymerase chain reaction.

tion of controls to prevent the spread of infections. We have successfully applied the PCR assay to lice from homeless and economically deprived persons in inner cities of developed countries and found high prevalences of *Bartonella quintana* infections (3,5,6). Furthermore, we have emphasized the risk of *R. prowazekii* outbreaks in Europe, based on our findings of an outbreak of epidemic typhus in Russia, a case of Brill-Zinsser disease in France (15), and a case of epidemic typhus imported from Algeria (9).

The PCR assay on lice may help detect outbreaks. In recent epidemics of louse-borne infections, the prevalence of body louse infestations in persons has reached 90% to 100% before clinical signs of louse-borne disease were noted in the population (16). Experience has shown that the emergence and dissemination of body lice can be very rapid when conditions are favorable (17). In Central Africa, large outbreaks of lice infections occurred during civil wars in Burundi, Rwanda, and Zaire (16) and preceded the outbreak of epidemic typhus by 2 years (7). We clearly demonstrate the potential for further outbreaks of louse-borne diseases in Africa. Although lice from Burundi were negative for *R. prowazekii* in 1998 and 2000 as a result of the administration of doxycycline to patients, the persistence of the vector enabled the spread of *R. prowazekii* from human carriers back into the louse population. In 2001, we found that 21% of lice from refugee camps in the same areas of Burundi as sampled earlier were positive by PCR for *R. prowazekii*. Further samples submitted to our laboratory indicate a typhus outbreak is currently developing in refugee camps in Burundi (unpub. data). We also found *R. prowazekii* in 7% of body lice collected in 2001 from a jail in Rwanda. That the country is now host to 300,000 refugees from the January 2002 eruption of the Nyiragongo volcano is thus a concern.

Although lice from the other areas studied were free from typhus, we found *B. quintana* to be widely distributed; it was detectable in lice from France, the Netherlands, Burundi, Zimbabwe, and Rwanda. We could not find the organism in lice from Australia, Tunisia, and Algeria, but only small numbers of lice from these areas were studied. As with *R. prowazekii,* chronic bacteremia occurs with *B. quintana* infection in humans; the only way to eradicate the organism is to eliminate body lice. We were not able to detect *Borrelia recurrentis* in any of the lice, which indicates that infection rates with this organism are very low or the agent is restricted to specific geographic zones.

Our study has demonstrated the usefulness of PCR of body lice in ongoing surveillance of louse-associated infections. When faced with outbreaks of body lice or to follow-up outbreaks of louse-borne infections, investigators should consider using PCR for *R. prowazekii*, *Bartonella quintana,* and *Borrelia recurrentis* in body lice collected from the study area and shipped to their laboratories. Our results from Burundi highlight the necessity for using combinations of methods to control body lice and hence *R. prowazekii* infections.

Dr. Fournier is a physician in the French reference center for the diagnosis and study of rickettsial diseases. His research interests include the physiopathologic, epidemiologic, and clinical features of rickettsioses.

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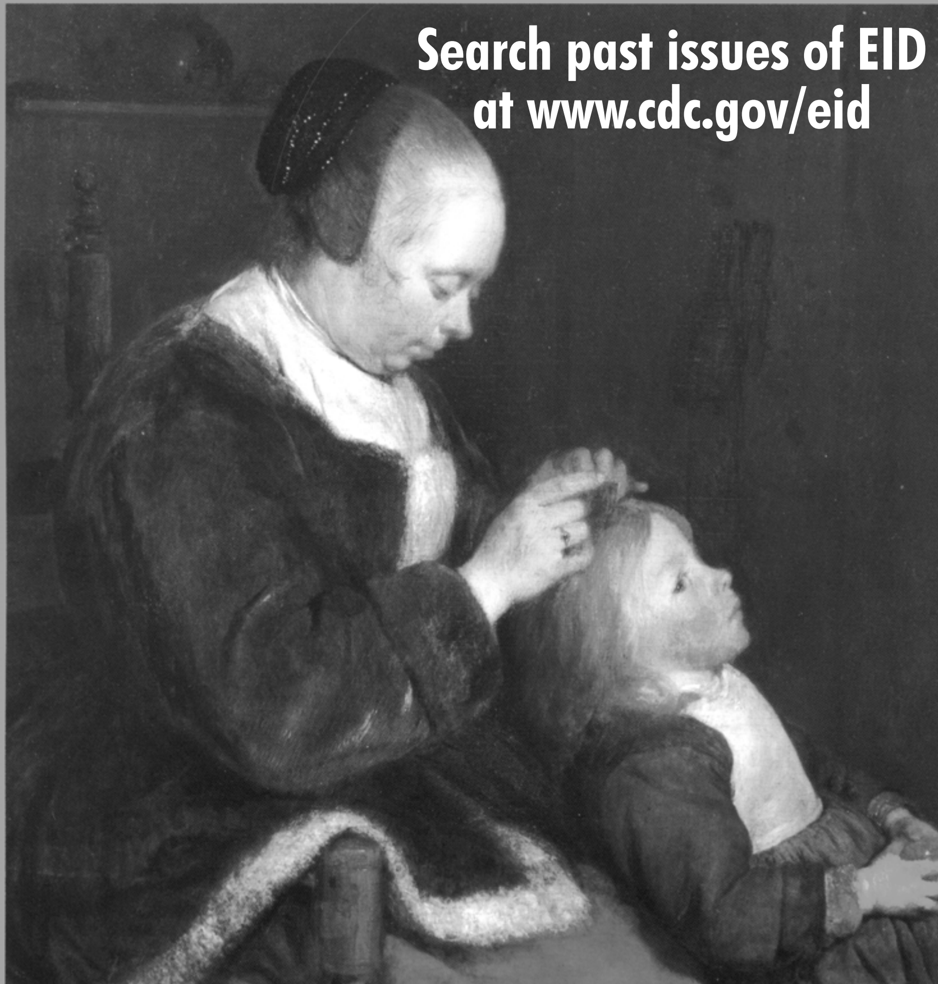

Clinical Failures of Linezolid and Implications for the Clinical Microbiology Laboratory

Brian A. Potoski,* Julie E. Mangino,* and Debra A. Goff*

Linezolid is the first in a new class of antimicrobials, the oxazolidinones. This antimicrobial is approved to treat gram-positive infections caused by vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) (1). Newer antibiotics have provided clinicians greater treatment options; however, ongoing experience shows limitations in their use. We report two patients with infections caused by linezolid-resistant and possible linezolid-nonsusceptible bacteria and provide commentary on the increasing bacterial resistance to linezolid and quinupristin/dalfopristin, as well as the appropriate use of these and future antibiotics.

Patient 1

A 47-year-old man's history was notable only for right ankle and subtalar arthritis. He underwent right ankle fusion with a nail placement in October 2000. In April 2001, he went to the emergency department for ankle pain secondary to a newly displaced fracture. The fusion nail was removed in the operating room; placement of a new longer nail was uncomplicated. One month later, he developed an ankle hematoma; it was drained, and he was given cephalexin for 7 days. Over the next several weeks, the area became erythematous and swollen; it spontaneously drained purulent material. The patient underwent irrigation and debridement on May 31, 2001. Gross pus was obtained and sent for Gram stain and culture. The patient was discharged with a prescription for amoxicillin/clavulanate tablets.

Culture grew *S. aureus* susceptible to vancomycin, trimethoprim/sulfamethoxazole (TMP-SMX), and gentamicin and resistant to all beta-lactams and clindamycin. Beta-lactams tested included penicillin, nafcillin, amoxicillin/clavulanate, cefazolin, and imipenem. Susceptibility testing of this MRSA isolate to linezolid was not performed. All testing was conducted by using a MicroScan (Dade Behring, Inc., West Sacramento, CA) automated system (unless otherwise noted) in accordance with National Committee on for Clinical Laboratory Standards (NCCLS) testing and quality-control recommendations (2).

Amoxicillin/clavulanate was discontinued, and oral linezolid, 600 mg twice a day, was begun. The patient did well, progressing to full weight-bearing capacity, and finished a 7-week course of linezolid. Two days after completing the course, nausea, fever, and chills developed, and he resumed linezolid. He went to the emergency room on July 30 with a temperature of 103°F; his ankle was warm and tender to palpation. Linezolid was stopped, and intravenous vancomycin, 1 g every 12 hrs, was started. The nail was surgically removed, Gram stain and cultures were obtained, and a peripherally inserted central catheter line was placed intraoperatively. He was continued on vancomycin. Cultures grew MRSA with the same sensitivities as the previous MRSA isolate. Additional susceptibility testing of this isolate to linezolid and TMP-SMX by E-test was performed; both were susceptible (MIC=4 µg/mL and 0.047 µg/mL, respectively). After 4 weeks, vancomycin was discontinued, and oral TMP-SMX, two double-strength tablets twice daily, was initiated for an additional 8 weeks of therapy. At followup, the patient's laboratory results showed a decreased leukocyte count, erythrocyte sedimentation rate, and C-reactive protein. He subsequently finished a total of 12 weeks of therapy and remained asymptomatic 6 months after completion of treatment.

Patient 2

A 41-year-old woman with refractory acute lymphocytic leukemia was admitted in May 2001 for an allogeneic bone marrow transplant. Her hospital course was complicated by *Klebsiella pneumoniae* sepsis, neutropenia, mental status changes, acute renal failure, and respiratory distress. She received several courses of antibiotics for extended periods including imipenem, amikacin, piperacillin/tazobactam, vancomycin, amphotericin B lipid complex, fluconazole, ciprofloxacin, and tobramycin. While she was receiving vancomycin, both peripheral and central venous catheter blood cultures grew vancomycin-resistant *Enterococcus faecium* (MIC >16 g/mL). The isolate was also resistant to other antibiotics tested (MIC of VRE was >8 µg/mL for both ampicillin and penicillin). Vancomycin was discontinued, intravenous linezolid, 600 mg every 12 hrs, was begun, and susceptibility testing to linezolid was requested. She remained on linezolid and died 3 days later. Subsequent susceptibility results revealed the isolate to be resistant to linezolid by E-test (MIC = 32 µg/mL). Confirmatory testing by broth dilution and E-test was performed at a reference lab, which verified resistance to linezolid and intermediate susceptibility to quinupristin/dalfopristin (MIC = 2 µg/mL).

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Patient 1, a pharmacist, preferred the convenience of oral linezolid therapy to intravenous vancomycin. Initial susceptibility of the MRSA isolate to linezolid was not obtained; however, a previous case report described success in treating MRSA and VRE bacteremia in a patient with MRI (magnetic resonance imagery)–confirmed vertebral osteomyelitis as the primary focus for infection (3). Although the second MRSA isolate in our patient was considered susceptible to linezolid by the NCCLS-defined MIC interpretive standard (MIC ≤4 µg/mL), the patient did not respond to a 7-week course of linezolid. Of interest, the MIC of MRSA to linezolid from our patient's isolate was 4 µg/mL, three dilutions greater than the isolate in the prior case report (MIC=0.5 µg/mL).

Patient 2's treatment was changed from empiric vancomycin to linezolid for VRE bacteremia without linezolid being part of her initial susceptibility data. Only after the patient died did the requested sensitivities to linezolid become available. Had linezolid been part of the initial susceptibility data, other therapies most likely would have been pursued since the isolate was resistant to linezolid. Arguably, her death might have been averted.

Multidrug-resistant organisms, especially VRE, continue to contribute to illness and death (4,5). In a published case series involving linezolid for 15 patients with VRE infections, mortality was noted to be very high at long-term followup (6). Two explanations might account for this high rate. First, the patients identified were severely ill with several coexisting conditions or illnesses. Second, other options were pursued before the initiation of linezolid, which may have inadvertently contributed to increased illness and death because of the delay in giving linezolid therapy. This latter argument is supported by studies (7,8) that show a significantly greater increase in hospital deaths if patients received inappropriate antimicrobial therapy compared to those whose initial therapy was appropriate.

These data, in addition to these recent experiences, have led us to routinely conduct susceptibility testing of linezolid and quinupristin/dalfopristin to VRE obtained from any sterile site. Previously, susceptibility data of linezolid and quinupristin/dalfopristin to VRE or MRSA were not routinely conducted unless specifically requested. Once the standard susceptibility panel was reported, if additional susceptibility tests were requested, they would be conducted with an E-test (AB Biodisk North America, Inc., Piscataway, NJ). This procedure was mainly because these drugs were not yet included on microtiter plates for commonly used automated systems (e.g., MicroScan, Vitek), and clinical reports of resistance were scarce. In addition, clinicians have a rather limited window of opportunity to request susceptibility testing to these newer agents because most bacterial isolates are saved for only 5 days. Our current practice includes obtaining tests of a VRE isolate's susceptibility to linezolid and quinupristin/dalfopristin as routine practice if the isolate is obtained from any sterile site. As experience and reports of resistance increase, this practice may guide appropriate therapy. Because of the large number of *S. aureus* isolates resistant to methicillin submitted to the microbiology laboratory annually, and because linezolid nonsusceptibility to clinical isolates of MRSA has only been reported once (9), we continue to conduct susceptibility testing of linezolid and quinupristin/dalfopristin to MRSA isolates only when requested.

To make the most appropriate use of these newer antimicrobial agents, data on susceptibility are needed. We recommend that tests for susceptibility to linezolid and quinupristin/dalfopristin be conducted before treatment is initiated. Although this procedure may no longer be necessary once standardized microtiter plates include these antibiotics, it is nevertheless relevant as clinical failures and reports of resistance mount. (4,9,10) Assuming antimicrobial sensitivity because an antibiotic is a recent addition to the antimicrobial armamentarium may lead to increased illness, deaths, and costs.

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Ebola-Poe: A Modern-Day Parallel of the Red Death?

Setu K. Vora and Sundaram V. Ramanan

Plagues and pestilence have evoked fear and awe since time immemorial. Often viewed as divine retribution, these scourges are mentioned in many cultural and religious texts, including the Bible, the Koran, and the Talmud. History itself is punctuated and shaped by epidemics, whose accounts are at the center of such literary works as Boccachio's Decameron, Daniel Defoe's A Journal of the Plague Year, and Gabriel Garcia Marquez' Love in the Time of Cholera. These works provide rare insight into the impact of real epidemics. Accounts of fictional epidemics, such as Albert Camus' The Plague or Edgar Allan Poe's The Masque of Red Death, are even more fascinating and debatable.

Poe's most famous works are macabre tales of terror, madness, decay, and death. The author's life has been the subject of numerous medical and psychiatric analyses, and the effects of alcoholism (1) and seizure disorder (2) on his creativity have been studied. Review of The Fall of the House of Usher has implicated porphyria (3) for the psychopathology of Roderick Usher and his sister Madeline. But to our knowledge, a detailed medical analysis of The Masque of Red Death has not been conducted.

The tale opens with the description of a mysterious epidemic. "The 'Red Death' had long devastated the country. No pestilence had ever been so fatal, or so hideous. Blood was its avatar and its seal—the redness and the horror of blood. There were sharp pains, and sudden dizziness, and then profuse bleeding at the pores, with dissolution. The scarlet stains upon the body and especially upon the face of the victim, were the pest ban which shut him out from the aid and from the sympathy of his fellow-men. And the whole seizure, progress and termination of the disease, were the incidents of half an hour."

To escape death, Prince Prospero secludes himself and a thousand noblemen in a castellated abbey. The epidemic rages and kills the poor who were left outside to fend for themselves. Six months into their successful bid to avoid the contagion, the callous prince and his friends celebrate within the sealed confines of the abbey, when quite suddenly the disease invades their sanctuary and kills everyone.

The Epidemiology

The story's opening line, "The 'Red Death' had long devastated the country," indicates that this is not a new disease outbreak but rather a known epidemic, ongoing or reemerging. High death rates leave Prospero's dominions "half depopulated." Poe gives a graphic description of the clinical features and outlines the course of the disease, from the earliest symptoms to the fatal outcome. The red death indiscriminately attacks all segments of the population, including healthy, immunocompetent hosts. Transmission, which seems to be accelerated by overcrowding, is from person-to-person contact and possibly aerosol inhalation. The death rate is highest in the abbey—all those confined inside die, while only half of those left outside do. Outside the abbey, once an infected person exhibits symptoms, others "shut him out from the aid and from the sympathy of his fellow-men."

Exercising literary license, Poe confers upon the prince and his courtiers an extended reprieve of 6 months (after they isolate themselves from the general population) before the disease strikes them down and they die in the span of only half an hour. If a few of the noblemen came into the abbey already infected, the symptom-free 6 months could be explained by a long incubation period. Conversely, if the prince and his companions were initially uninfected, the microbe could have gained entry into the fortified sanctuary at a later time and caused the explosive epidemic. Even the strong physical barriers surrounding the abbey could not have stopped airborne or vector-borne transmission.

The geographic location of the story also offers clues about the epidemic. The presence of a prince and knights suggests a royal and feudal structure of governance somewhere in Europe. The use of "improvisatori" (from Italian *improvisatore*, an entertainer who improvises verse) (4) for courtly entertainment during their isolation suggests that the setting of this tale is Italy or a nearby European country.

Possible Models for the Red Death

Even though a fictional product of Poe's fertile and bizarre imagination, the red death is likely modeled after a disease within the author's lifetime and experience. Some have speculated that Poe's family history of tuberculosis (his mother, his adoptive mother, his wife, and possibly his brother died of the disease) may have prompted him to write about a similar disease in Life in Death—a story about a painter and his dying wife, who incidentally resembled Poe's wife (5). Along the same lines, Poe's experience of nursing his wife through her bouts of exsanguinating hemoptysis, cradling her head for hours, and wiping away the blood from her face may well have been on his mind when he mused about "the scarlet stains upon the face" of the afflicted in Masque of the Red Death.

As described by Poe, the red death seems to be some type

of a viral hemorrhagic fever. Epidemics of yellow fever killed 100,000–150,000 in the United States from 1693 to 1905 (6). Northern ports (Boston, New York, Philadelphia, Baltimore), where Poe lived at various times in his life, were affected by yellow fever until 1822. He could have been inspired by a nationwide, severe epidemic of yellow fever in 1841 (a year before he wrote The Masque of Red Death), but yellow fever was a commonplace disease without any mystery attached to it. Like red death, yellow fever causes high body temperatures; body ache; damage to capillaries, which can result in bleeding from the nose and mouth; stools stained dark with blood; and (the most dreaded symptom) copious black vomit caused by gastric bleeding. Poe's red death, however, has a much higher death rate and communicability. Besides, the eponymous jaundice of yellow fever is not described as a feature of red death. Poe named his fictional disease "red" death, probably to differentiate it from "black" death, otherwise known as the plague. "Red" death is also descriptive of the profuse bleeding characteristic of this fictional disease. Poe maximizes the horror of the disease by intentionally making it mysterious and universally fatal. By alluding to the black death, he invokes memories of the vast plague epidemics that ravaged the world.

The Diagnosis

Poe's description of the red death is in line with the clinical features of filovirus hemorrhagic fevers, which include Ebola and Marburg (7). Viral hemorrhagic fever outbreaks are rare but not unheard of in Europe. Crimean-Congo hemorrhagic fever and Marburg hemorrhagic fever have occurred in Europe. So, it is plausible for a filovirus to strike in Europe, which seems to be the setting of the red death. However, writing in 1840, Poe could not have known about Ebola or Marburg.

A potential, but unlikely, cause for the disease that Poe calls red death is some type of Marburg-like virus. During the self-imposed seclusion, Prospero provides his guests courtly entertainment in the form of buffoons, improvisatori, and ballet dancers. On these occasions, live animals were part of the program—we see them in the works of European artists who painted scenes from contemporary life. Peitro Longhi, a reputed Venetian artist, recorded the arrival of exotic animals at Carnevale in his painting Rhinoceros (in 1751) (8). The emblem of Carnevale was masked festivity, such as the one in Poe's story. It is easy to conjure up monkeys imported from colonies in Africa to perform antics and entertain the princely court. The monkeys could have triggered a Marburg-like hemorrhagic fever—as seen at some European laboratories in recent times (9). However, this scenario requires the presence of African monkeys, which Poe does not mention. Besides, although this scenario could explain a contained outbreak in the abbey, it does not account for the major epidemic in the general population.

Filoviral diseases begin with abrupt onset of fever usually accompanied by myalgia. Poe's description of "sharp pains" is eerily prescient. In the 1976 outbreak of Ebola in Sudan, knife-like sharp chest pain was an early symptom (10). Central nervous system involvement, also a feature of Ebola fever, may account for the "sudden dizziness" and delirium seen in red death. The prince and his courtiers chase the spectral imagery of the red death, and "gasp in unutterable horror at finding the grave-cerements and corpse-like mask which they handled with so violent a rudeness, untenanted by any tangible form." These images of red death were evidently hallucinations. "Profuse bleeding at the pores" and "scarlet stains upon the body and face" of infected persons resemble the petechiae, ecchymoses, and mucous membrane hemorrhages found in half or more of Ebola fever patients.

Like outbreaks of the red death, Ebola outbreaks remain shrouded in mystery, in spite of modern technologic investigations. Ebola's origin and natural reservoir, as well as its location and behavior between outbreaks, remain poorly understood. Different strains of the virus have different death rates, with the Ebola- Zaire strain being the most lethal. Poe's red death brings to mind some form of Ebola-like viral hemorrhagic fever of extremely high virulence.

Whether inspired by tuberculosis or yellow fever, the red death is clearly a concoction of Poe's imagination. In honor of the creative genius that imagined Ebola fever long before the infection was recognized, the particular strain that causes red death might be named Ebola-Poe.

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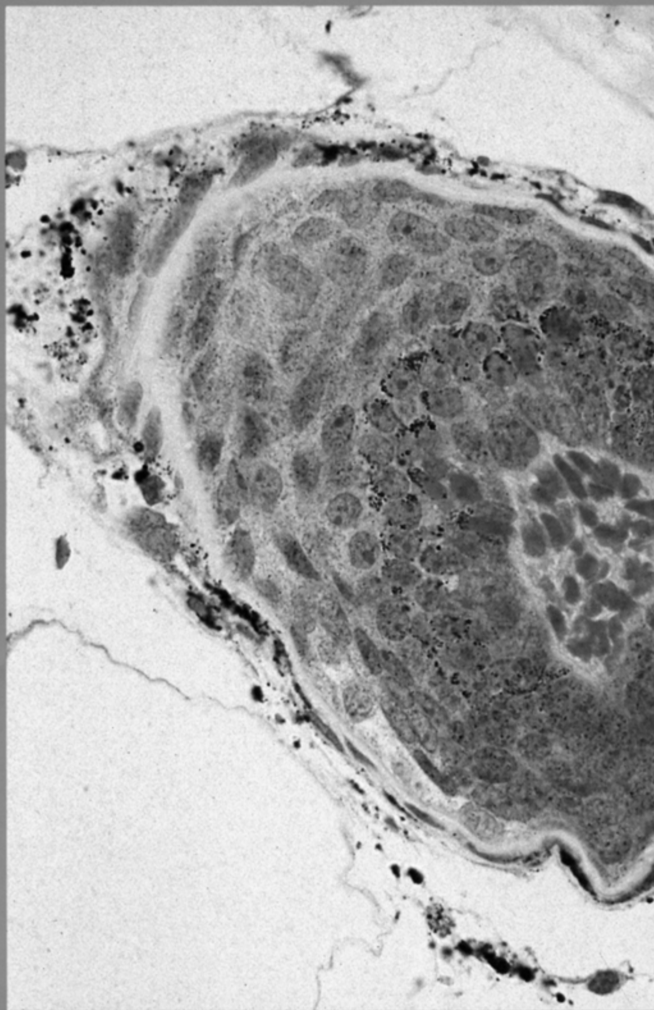

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Avian Reservoirs of the Agent of Human Granulocytic Ehrlichiosis?

To the Editor: Human granulocytic ehrlichiosis (HGE), first described in 1994 (1), is the second-most common tick-borne disease in the United States; Lyme disease is the most prevalent. Both diseases are transmitted by blacklegged ticks (*Ixodes scapularis* Say) that are abundant in southern New York state (2). Factors that influence the risk for infection, particularly the role of wildlife in transmitting the etiologic agent, *Anaplasma* (formerly *Ehrlichia*) *phagocytophilum* (3,4), to vector ticks are not well understood. In the absence of transovarial transmission (5,6), acquisition of *A. phagocytophilum* by its vector must result either from feeding on the blood of reservoir-competent hosts or by cofeeding of uninfected ticks in close proximity to infected ticks (7).

The role that birds may play in HGE ecology is potentially very important. Birds in the northeastern United States routinely host immature *I. scapularis* (8), and several species are competent reservoirs of *Borrelia burgdorferi* (9), the causative agent of Lyme disease, thus providing an opportunity to transport infected ticks to new areas. While data for HGE are lacking, *A. phagocytophilum*–infected *I. ricinus* nymphs have been collected from migrating birds in Sweden (10); reservoir competence was not established, however. Our goal was to determine if several common bird species might serve as reservoirs of *A. phagocytophilum* and, as such, transmit the pathogen to feeding *I. scapularis* larvae.

Birds were sampled at a deciduous woodland preserve in Tarrytown, Westchester County, New York, an area where Lyme disease and HGE are endemic. Birds were captured with Japanese mist nets (#3, EBBA Net Committee, Bryn Athyn, PA), and all captures were examined for ectoparasites. Ticks were removed with forceps and preserved in 70% ethanol for later identification and testing. Birds were then banded and released unharmed. Host-seeking larvae also were collected by drag sampling at the site to further evaluate the potential for transovarial transmission of the HGE agent. To ensure that all larvae were not from a single egg mass, no more than 25 specimens were collected from a single drag.

A total of 123 larval *I. scapularis* were collected from birds captured in May 1993 (one American robin [*Turdus migratorius*] hosting two larvae), July 1997 (one American robin hosting 31 larvae), and August 1997 (two veerys [*Catharus fuscescens*] hosting 10 and 42 larvae, respectively; one wood thrush [*Hylocichla mustelina*] hosting 19 larvae; and one rose-breasted grosbeak [*Pheucticus ludovicianus*] hosting 19 larvae). Additionally, 10 *I. scapularis* nymphs were collected from five of the six birds: 5 and 2 nymphs, respectively, from the robins and a nymph each from the veerys and grosbeak.

Tick infection with *A. phagocytophilum* was determined by polymerase chain reaction (PCR) amplification of a 16S rDNA-associated DNA region specific for this organism. Nymphal ticks were tested individually, and larval ticks were analyzed in pools of five and six individuals; all ticks in a pool were from a single bird. Ticks were minced with a sterile 18-gauge needle in sample buffer provided in the Isoquick DNA extraction kit (ORCA Research, Bothell, WA); DNA was isolated from samples according to the manufacturer's directions, as described (11). Strict procedures to prevent cross-contamination or carryover of amplified products were employed throughout, as described (11). For *A. phagocytophilum*, a 537-bp PCR product was obtained in a nested PCR reaction protocol employing primers 16S UniF/23S UniR (16S UniF 5´-GAAGTCGTAA-CAAGG-3´ and 23S UniR 5´-GCCA AGGCATCCACC-3´) in the first reaction, and primers 16S NF/23S NR (16S NF 5´-GTAGGTGAACCTGCGG-3´) and 23S NR (5´-CCAGTGTAAAATA CTCTTTCC-3´) in the second round. This PCR protocol resulted in amplification of an appropriately sized product from *E.* *equi* (PRO Synon. *A. phagocytaphilum)* strain MRK, six cultured clinical isolates of *A. phagocytophila* from HGE patients, and 16 field-collected ticks. No PCR product was obtained from either *E. chaffeensis* or *E. canis* as templates (Liveris and Schwartz, unpub. data). Three clones of each PCR product were randomly selected for sequencing in both the forward and reverse directions. DNA sequence data were collected, edited, and compared to known *A. phagocytophilum* sequences on the BLAST server at the National Center for Biotechnology Information (NCBI) web site (available from: URL: http://www.ncbi.nlm.nh.gov). For *B. burgdorferi*, a 941-bp region of the 16S-23S rDNA spacer was used as a target for amplification using a nested PCR procedure (12).

Of the 25 larval pools, 5 (20%) tested positive for *A. phagocytophilum*—three pools from a single veery and two pools from a robin. This percentage suggests a minimum infection rate of 4.1% (5/123). In contrast, none of 300 field-collected larvae, comprising 30 pools of 10 larvae each, were PCR positive. This difference is significant (Arcsine transformation of percentages and test of equality; t_s = 3.81, $p<0.001$). PCR products from one of the *A. phagocytophilum*–positive pools obtained from the robin and from two pools from the veery were cloned and sequenced. All sequences were identical except for a single nucleotide change in the product from the veery. The sequence derived from the tick larvae recovered from the robin was identical to those obtained for the MRK strain of *E. equi* and six *A. phagocytophilum* isolates obtained from HGE patients (Liveris and Schwartz, unpub. data).

Three (12%) of the 25 larval pools were positive for *B. burgdorferi* (one

pool each from the wood thrush and both robins). In addition, one of the larval pools from a robin was PCR positive for both *A. phagocytophilum* and *B. burgdorferi*. Thus a single robin may have been coinfected with the causative agents of HGE and Lyme disease and transmitted them to feeding ticks. None of the attached nymphs was positive for either agent, suggesting that larval infection resulting from cofeeding did not occur as a result of feeding in proximity to these nymphs.

These data suggest that at least two species of birds, the American robin and the veery, may be reservoirs for *A. phagocytophium* by infecting larval *I. scapularis* as they feed. Both bird species are reservoirs for *B. burgdorferi* (9), and robins appear to be capable of maintaining and transmitting both the agent of HGE and that of Lyme disease concurrently. This phenomenon has been reported for mammal-feeding *I. scapularis* (13) but not previously for bird-feeding ticks.

Although none of the nymphal *I. scapularis* attached to birds concurrently with larvae showed evidence of *A. phagocytophilum* or *B. burgdorferi* infection, larvae may have acquired both agents by cofeeding with nymphs that detached before capture of the birds. Further work is needed to exclude that possibility.

Despite small sample sizes, relatively high levels of *A. phagocytophilum* transmission occurred in this study. Because *I. scapularis* larvae remain attached for several days while feeding, replete newly infected larvae may be deposited miles from where they first encountered the host. This pattern effectively seeds new sites with potential vectors of two tick-borne pathogens. However, confirmation that birds can serve as reservoirs of *A. phagocytophilum* is needed, preferably through studies of a wide range of avian species. To date, the number of bird species for which adequate numbers have been captured and from which sufficient numbers of ticks have been collected and tested to determine reservoir status, is quite low. Even in cases where sufficient numbers of birds are collected (10), very low tick loads, a host's immune response, or both may contribute to low infectivity. In particular, additional work should be conducted in areas where Lyme disease and HGE are endemic. By identifying reservoir-competent bird species and understanding movement patterns that include seasonal migrations covering large distances, the spread of *A. phagocytophila* and the resulting risk that may face human residents in new foci can be clarified.

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Emerging Leptospirosis, North India

To the Editor: We read with interest the article, The Changing Epidemiology of Leptospirosis in Israel, published in volume 7, no. 6 (1). Leptospirosis, a septicemic zoonosis with multisystemic involvement, is caused by the pathogenic strains of *Leptospira interrogans*. Rural farm workers are at high risk for leptospirosis, and it can be a significant public health problem when water and food safety are not ensured. Several epidemics of leptospirosis have occurred on Andaman and Nicobar islands and in southern and western parts of India during the past century (2). The organism has been detected in farm animals in many parts of the country (3); however, human infections have been more or less localized. In 1998, researchers warned that, unless adequate public health measures were initiated, large leptospirosis epidemics were possible in areas where the disease had not been previously reported (4). In addition, they recommended improving clinical diagnosis and conducting systematic epidemiologic studies for control of the disease (4).

The true incidence of human leptospirosis in northern India is not known either because of a lack of awareness on the part of the treating physicians or the lack of diagnostic techniques. In 1966, human leptospirosis was reported in Delhi, a state in northern India (5). In a 1966 study (5), sera from persons with pyrexia and jaundice were tested by the agglutination lysis test for leptospiral antibodies. Of 93 serum specimens from persons with pyrexia cases, 3 were positive (1 with *L. icterohemorrhagica* and two with *L. canicola*); of 43 serum specimens from persons with jaundice, 3 were positive (2 with *L. icterohemorrhagica* and 1 with *L. icterohemorrhagica* and *L. pomona*). No other study on leptospirosis has been done in the region, and no data are available concerning the problem.

To assess the current status of transmission in Delhi and its adjoining areas, we conducted a systematic study for the diagnosis of leptospirosis in our hospital from April 2000 to March 2001; case definition criteria suggested in a previous study (4) were used. A case was defined as a person with fever, headache, and myalgia and more than two of the following symptoms: jaundice, oliguria, respiratory symptoms (cough, hemoptysis, and breathlessness), hemorrhagic manifestations (hematemesis, bleeding gums, and subconjunctival hemorrhage), and signs of meningeal irritation and convulsion. Seventy-five patients (44 male patients; 3–73 years of age) satisfied the inclusion criteria. In addition to clinical evaluation and assessment for other diseases, leptospirosis was investigated by the following laboratory methods: isolation of *Leptospira interrogans*, direct visualization of the organism under dark-field microscopy, and enzyme-linked immunosorbent assay (ELISA) for *Leptospira* immunoglobulin (Ig) M antibody (Serion Immunodiagnostica GmbH, Würzburg, Germany). Per manufacturer's specifications, the sensitivity, specificity, positive predictive value, and negative predictive value of this kit are 96%, 97%, 90%, and 99%, respectively). All blood samples were sent to the *Leptospira* referral laboratory at the Indian Veterinary Research Institute, Izzatnagar, for microscopic agglutination test (MAT). Eight serovars of *L. interrogans* (*australis, autumnalis*, *pomona, sejroe, tarassovi, icterohaemorrhagica, hebdomadis,* and *patoc*) were tested, and a agglutination titer of more than 1:100 was considered positive. All patients were treated empirically with broad-spectrum antibiotics as well as specific drugs according to the results of investigations.

Thirty-two patients (42.6%) had a positive ELISA test for *Leptospira* IgM antibody. The results of MAT were positive in 21 (65.6%) of the 32 ELISA-positive serum samples. Serum specimens from 11 patients reacted with a single serovar, and specimens from 10 patients reacted with more than one serovar. Among the pathogenic species, *Leptospira* antibodies were detectable by MAT predominantly against *L. sejroe* (7 of 21), followed by *L. icterohaemorrhagica* (6 of 21), *L. hebdomadis* (4 of 21), and *L. tarassovi* (4 of 21). *Leptospira* antibodies were also detectable against *L. autumnalis* (3 of 21), *L. australis* (2 of 21), and *L. pomona* (1 of 21). Against *L. patoc*, MAT could detect antibodies in six samples. The organism could not be isolated in culture or visualized under dark-field microscopy in any of the specimens. Of the 43 case-patients with ELISA-negative specimens, alternative diagnoses were established for 40 on the basis of various laboratory investigations. In five of the patients with ELISA-positive specimens, coinfection with other pathogens was detected, including *Salmonella typhi* (one case) by a positive Widal test, hepatitis C virus by positive ELISA (two cases), and *Plasmodium falciparum* (two cases) by a positive smear. Five patients, including three who were ELISA positive for *Leptospira*, died. The highest number of ELISA-positive serum samples (21 of 32) were obtained in August and September 2000, suggesting an epidemic.

Epidemiologic investigation of leptospirosis is often hampered by the difficulty of making a definitive microbiologic diagnosis. Isolation of leptospira from clinical samples provides a definitive diagnosis; however, the value of culture is limited because samples have to be collected before the administration of antibiotics, and culturing requires prolonged incubation. Demonstration of typical motility of leptospira under dark-ground illumination in clinical samples, though helpful in early diagnosis, has low sensitivity and depends on the technician's opinion. Measurement of IgM antibodies against *Leptospira* by ELISA has emerged as a reliable diag-

nostic test with good specificity and sensitivity (6). The probability of achieving a positive serologic test increases with the duration of disease, and good correlation between results of MAT and ELISA has been reported by Cumberland et al. (7). MAT has emerged as a dependable diagnostic tool for leptospirosis (next to isolation) by providing serovar specific diagnosis. However, a large number of serovars of *L. interrogans* exist, and maintaining large numbers of organisms for MAT is difficult for most laboratories. Moreover, MAT may fail to detect antibodies when specific serovars are not used. In this study, the ELISA-positive samples, for which MAT results were negative, may have been caused by infection with serovars other than those used in this study. Because of the problems with methods, leptospirosis is grossly underdiagnosed.

Leptospira organisms require humid weather for their survival. Rodents and domestic animals (i.e., cattle and dogs) harbor leptospires and shed the bacteria in urine; they may disseminate the organism to rain water and drinking water sources. Humans frequently come in contact with contaminated water during floods; the number of cases is higher during and after heavy rainfalls. We found that the peak incidence of the disease was during August and September, the monsoon season, which may explain the high incidence of seropositivity during this period. Though the organism has been detected in farm animals in northern India, human leptospirosis has not been considered a major public health problem, probably because transmission is low in arid weather conditions. As a result of 13 consecutive monsoons of above-average strength in India, changes in the environment may be promoting the transmission of this organism. Recently, two other regions in northern India, Chandigarh (8) and Varanasi (9), have reported a *Leptospira* seroprevalance rate of 8.8% and 21.74%, respectively.

Our study supports the warning from other researchers regarding the threat of leptospirosis in areas such as northern India. Preventive measures should be initiated and rapid and definitive diagnostic tests must be developed.

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James A. Ferguson Emerging Infectious Diseases Fellowship

The Office of Minority and Women's Health, National Center for Infectious Diseases, Center for Disease Control and Prevention (CDC), announces the James A. Ferguson Emerging Infectious Diseases Fellowship Program, 2003.

This fellowship program is an 8-week professional development experience for racial and ethnic minority students in medical, dental, veterinary, pharmacy, and masters of public health graduate programs. Fellows participate in a broad array of public health activities. The program is administered through a cooperative agreement between the Minority Health Professions Foundation and the National Center for Infectious Diseases, CDC. Fellows are paired with a mentor based on their statement of interests and qualifications. They are required to prepare and present a formal scientific presentation on their work to CDC scientists and staff at the end of the program and to submit a formal research paper. The students receive stipends, housing, and transportation to and from Atlanta.

The program is designed to increase the students' knowledge of public health and public health career paths and to introduce fellows to careers addressing infectious diseases and racial and ethnic health disparities. The ultimate goal of the program is to influence students to pursue careers in public health and specific disciplines needed by the National Center for Infectious Diseases to strengthen and diversify the workforce.

The deadline for submitting applications for this fellowship is February 28, 2003. For additional information about the program, please contact Edith A. Hambie at eah1@cdc.gov, or call 404-371-5310.

Book Review

The Microbial Challenge: Human-Microbe Interactions

Robert I. Krasner
American Society for Microbiology Press, Washington, 2002
ISBN: 1-55581-2414
Pages: 433
Price: $89.95

During the past decade, a wide array of books have addressed the topic of new and emerging infectious diseases. In The Microbial Challenge: Human-Microbe Interactions, Robert I. Krasner attempts to put this topic into a format appropriate for classroom presentation. The book is sufficiently well done to stand alone in some situations, but supplemental material may be necessary in others.

The Microbial Challenge is relatively compact, compared with the usual microbiology textbook, with expected advantages and unavoidable shortcomings. In 400 pages and 16 chapters, the author captures basic principles of microbiology, immunology, epidemiology, and infectious diseases, including chapters on biological weapons, "current plagues," and factors underlying emergence of infectious diseases. A chapter on the control of microbial diseases is not unexpected, but the subsequent chapter on partnerships in the control of infectious diseases is an unusual and welcome addition. Chapters are organized into three major parts: "The Challenge," "Meeting the Challenge," and "Current Challenges." However, these designations reflect the author's perspective more than specific content. Similar chapters in other texts might simply be categorized under the generic theme "microorganisms and humans."

The topics and organization in the book comprise characteristics well suited for a textbook. Each topic is presented clearly and succinctly. The book has many wonderful photographs and illustrations that bring the text to life, including unique photographs from field situations (many from the author's personal archives). These photographs demonstrate that microbiology and infectious diseases extend far beyond the hospital and laboratory. A series of self-evaluation questions follow each chapter. Students will find the glossary quite useful. The author also provides a brief list of websites that might be helpful to instructors.

Although this book provides an informative summary of principles that are quite useful for a survey course on microbiology and infectious diseases, the abbreviated treatment might prove unsatisfactory for some instructional situations. For example, do not expect to find the detailed descriptions of chemical principles, central metabolic pathways, DNA replication, transcriptional control of gene function, or protein and cell-wall synthesis found in standard (and much larger!) textbooks. Taxonomy and diagnostic microbiology are only briefly discussed. The focus on microorganisms and diseases at the expense of such details may be an asset or a liability, depending on your perspective.

The author anticipates that the book would be suitable for a course without a laboratory component but suggests that certain exercises be added from other sources, as needed. The decision not to include a laboratory component is a critical departure point for the book's utility. Although the text would be quite suitable in some curricula for a survey course on microorganisms and infectious diseases, the failure to include principles and applications of molecular biology seems a serious omission, especially for students who go on to graduate school and pursue careers in laboratory-based research. Indeed, the only surprise I had in reviewing the book was the lack of discussion of molecular techniques because they are commonly used to identify and subtype pathogens during epidemiologic investigations.

The Microbial Challenge could well serve audiences beyond the undergraduate level. The text would be an excellent choice for schools of public health, provided that students are given supplemental readings and detailed case studies for analysis. Considering the current interest in infectious diseases and bioterrorism, this book would be a useful resource for government staff at the national and local levels.

Joseph E. McDade

Rome, Georgia

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Conference Summary

Institute of Medicine Forum on Emerging Infections: Linking Infectious Agents and Chronic Diseases

The belief that many long-recognized chronic diseases are infectious in origin dates to the mid-nineteenth century, when cancer was studied as a possible infectious disease. In the 1950s and 1960s, much biomedical research was unsuccessful in confirming microbial causes of various chronic syndromes. Recent years, however, are marked by successful identification of several causal infectious agents of chronic disease such as *Human papillomavirus* in cervical cancer, but challenges and controversies remain.

The Institute of Medicine's Forum on Emerging Infections recently sought to address this rapidly evolving field. To identify cross-disciplinary contributions and challenges in determining infectious causes of chronic diseases, the forum hosted a 2-day workshop on October 21–22, 2002, Linking Infectious Agents and Chronic Diseases: Defining the Relationship, Enhancing the Research, and Mitigating the Effects. In response to invited presentations, participants explored factors driving infectious causes of chronic diseases to prominence, identified difficulties in linking infectious agents with chronic conditions, and discussed broad-based strategies and research programs that might advance the field.

Invited experts provided research findings on a diverse range of recognized and potential chronic sequelae of infection as well as diverse pathogenic mechanisms from exposure to chronic outcome. Cancers, demyelinating syndromes, cardiovascular disease, neuropsychiatric diseases, hepatitis, and diabetes mellitus were among the chronic conditions addressed. Ensuing discussions noted gaps in knowledge and in the translation of research data to health-care interventions for both accepted and speculative causal associations. Workshop participants remarked on the likely widespread clinical and public health implications of linking infectious agents with chronic diseases that dominate health care in economically established countries, including the United States. The potential benefits of detecting and preventing causal infections, and the risks of interventions against unproven causal agents, are substantial. Workshop participants advocated careful research to produce and appropriately translate validated, reproducible data into clinical management to alleviate the impact of chronic diseases.

Participants also recognized the potential impact of infectious disease control on chronic diseases in economically developing countries. Within 20 years, chronic diseases are expected to represent a substantial proportion of their health burden. Presentations on human T-cell lymphotropic virus type 1 infection and hepatitis C–schistosomiasis coinfection demonstrated the impact of progressive chronic infections that disproportionately affect developing regions. These presentations emphasized the importance of considering coinfections in chronic disease pathology. Data on chronic outcomes of malaria in infected persons and unborn children and of other coinfections emphasized these points. Presentations also examined causal associations between enteric or parasitic infections and long-term developmental disabilities, as well as links between infectious agents and epilepsy. Coinfections and common acute infections may represent an under-recognized source of chronic pathology. In regions with limited health-care resources, newly identified infectious causes of chronic diseases, including tuberculosis and malaria, may require increased attention.

Against the backdrop of multiple microbes and multiple chronic outcomes, participants attempted to identify research opportunities, challenges, and barriers to understanding linkages between infections and chronic syndromes, and ultimately efforts to mitigate the impact of chronic diseases on human health. Recent developments in technology, methodology, and collaborative research have clearly advanced the ability to determine causal relationships. However, the workshop highlighted numerous factors that complicate identification and confirmation of one or more infectious roots of a chronic disease—factors that current and future research must address. These challenges include possible multifactorial pathogenesis such as interactions between environmental and genetic (host and microbe) influences; how the timing of infection determines final chronic outcome; and the "hit-and-run" nature of certain microbes that may be eliminated before chronic disease becomes apparent. Additional challenges include differentiating the roles of acute, persistently active, latent, and recurrent infection in pathogenesis; the possible singular role of certain species or strains in producing chronic sequelae; the influence of coinfections in defining final pathology; difficulty detecting latent infection before or when chronic disease is diagnosed; differences in the sensitivity and specificity of detection assays in different tissues; difficulty culturing certain microbes; and the lack of adequate methods to identify novel or rare microbes, viruses, and other pathogens. Equally complex is balancing investment in potential infectious causes of multifactorial, high burden diseases with that for rarer conditions that may have one primary cause, infection.

Participants noted that recently developed molecular and immunologic techniques (e.g., representational difference analysis, gene-chip profiling of host and microbe, immune

response profiling, proteomics) offer new ways to overcome several obstacles to identifying potential etiologic agents. However, continued investment in new technologies or improving existing methods remains key to overcoming challenges. Defining the temporal relationship between infection and chronic disease with appropriate technology is critical to translating science into effective clinical strategies that intervene against infection to prevent or minimize chronic disease.

Discussion further emphasized that scientifically sound, new technologies must be applied to and guided by a foundation of epidemiologic clues from well-designed studies and surveillance systems. A multipronged approach will be critical. Research and public health activities need to facilitate appropriate linkage of existing and newly designed databases, ensuring quality surveillance and epidemiology that better characterize infectious and chronic diseases with their distributions and potential associations. Many settings demand longitudinal investigations to complement case-control or cross-sectional studies, requiring longer term investment. Detecting and confirming causal associations will require study of both larger cohorts and at-risk subpopulations.

Improved coordination between basic and clinical scientists, pathologists, and epidemiologists is critical to these goals. Networks and collaborative teams are needed to develop and demand the necessary standardized case definitions (for the infection and the chronic outcome), new and adequate specimen collections with pedigree databases, and comparable methods of analysis. Overall discussions emphasized two major themes of the workshop: 1) the need to define the nature and scope of future research that balances global efforts among the various chronic syndromes and 2) development of a coordinated and systematic strategy to maximize resource use and overcome the inherent technologic, epidemiologic, and organizational challenges in this field.

A published summary of the workshop that includes individually authored papers by workshop presenters will be available in early 2003 from the National Academy Press (available from: URL: www.nap.edu or 800-624-6242). Additional information about the activities of the Forum on Emerging Infections can be found at URL: http://www.iom.edu under the Board on Global on Health or by contacting 202-334-3992.

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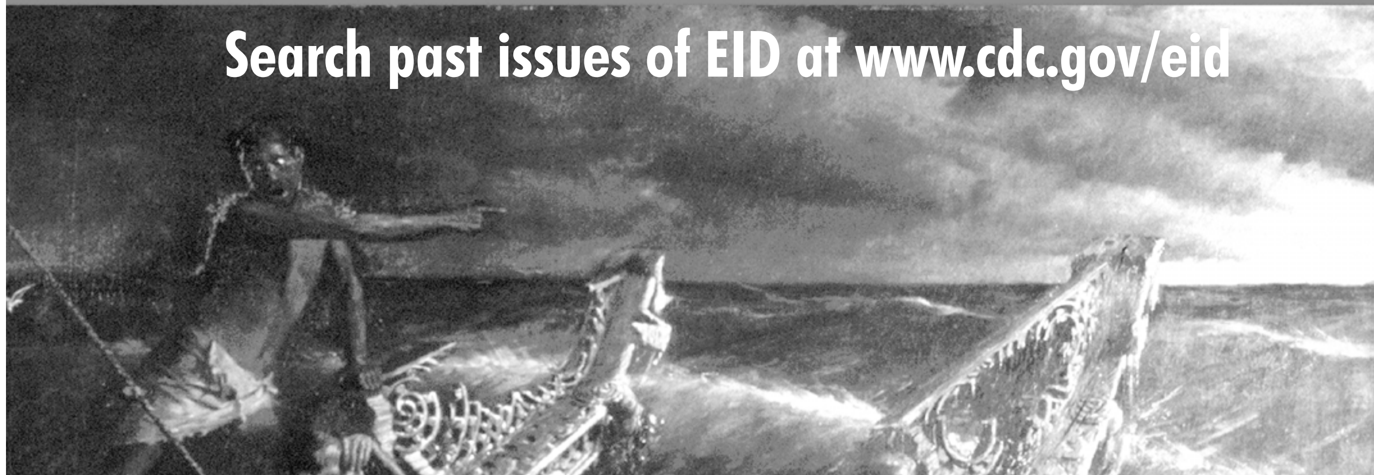

About the Cover

Giotto di Bondone (c. 1267–1337).
St. Francis of Assisi Receiving the Stigmata (c. 1290).
Tempera on wood, 313 cm x 163 cm. Musée du Louvre, Paris, France

Giotto di Bondone, founder of the Italian school of painting, was born in a village near Florence. Legend has it that Cimabue, the great master of the late 13th century, found Giotto herding sheep in the Italian countryside, noticed that the youth was drawing one of the sheep on a rock, and took him under his tutelage. Even though not much is known about Giotto's life, his era of the great cathedrals was a time of artistic renaissance in Italy. While some argue that Giotto did not single-handedly create the remarkable artistic developments of his time, few dispute that these developments reached their peak in his work (1).

Giotto is considered the first painter in the history of Western art to place human figures within realistic surroundings. Moving away from Byzantine tradition, he introduced pictorial space, which strengthened figures, giving them three-dimensional force and structural importance. With a keen eye for detail, he painted characters in all walks of life, from peasants and townspeople, to mystics and popes. Giotto was recognized as an artist in his lifetime and was said to have "translated the art of painting from Greek to Latin" (2).

In 1296, Giotto was invited to paint the story of St. Francis of Assisi in 28 scenes for the famed San Francesco basilica in Pisa. This work, Giotto's best, introduced the principles of actuality and reality in painting and was the earliest example of the Italian school. The painter drew from historical accounts of the life of St. Francis to create a likeness of the saint that was intense, realistic, and reminiscent of every day life—a complete departure from the stylistic and symbolic. The image of St. Francis was set in a topographic context highlighting the architectural and physical surroundings (1).

The rural scene featured on this cover of Emerging Infectious Diseases depicts St. Francis' sermon to the birds. The scene lovingly merges the surroundings (the trees and the birds) in a harmonious composition of human activity (and divine presence) in perfect tune with nature—nature elevated in importance and set in the forefront. The monk, wearing a luminous halo, is engaged in a commonplace interaction with an odd collection of birds, humble creatures that nonetheless seem to appreciate the mysterious circumstance: the miracle of the stigmata (the marks, according to religious tradition, of Christ's suffering).

In this depiction of St. Francis, Giotto's genius lies not only in the skillful juxtaposition of the realism in the scene (contemporary attire, animals, trees, the monk) with notions of the miraculous, but also in the enlightened awareness of the world as a structured composite in which all the pieces, human or not, contribute to the integrity of the whole. Whether (as in the 13th century) they observe with rapt attention the stigmata of suffering or (as in our times) themselves carry the stigmata in the form of disease, the birds are clearly an integral part of the scene.

Polyxeni Potter

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2. Laclotte M, Cuzin J-P. The Louvre: European Paintings. Lond Publications Ltd.; 1993.

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Upcoming Issue

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

Look in the January issue for the following topics:

Congenital Transmission of *Trypanosoma cruzi* Infection in Argentina

Epidemic Hand, Foot and Mouth Disease Caused by Human Enterovirus 71, Singapore

Molecular Surveillance System To Track Global Patterns of Drug Resistance to Malaria

Natural Enzootic Vectors of Venezuelan Equine Encephalitis Virus, Magdalena Valley, Colombia

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Highly Endemic, Waterborne Toxoplasmosis in North Rio de Janeiro State, Brazil,

Shiga-Toxin–Producing *Escherichia coli* Infections Associated with Hemolytic Uremic Syndrome, Italy, 1988–2000

Foot and Mouth Disease and Cryptosporidiosis: Possible Interaction between Two Emerging Infectious Diseases

Persistence of W135 *Neisseria meningitidis* Carriage in Returning Hajj Pilgrims: Risk for Early and Late Transmission to Household Contacts

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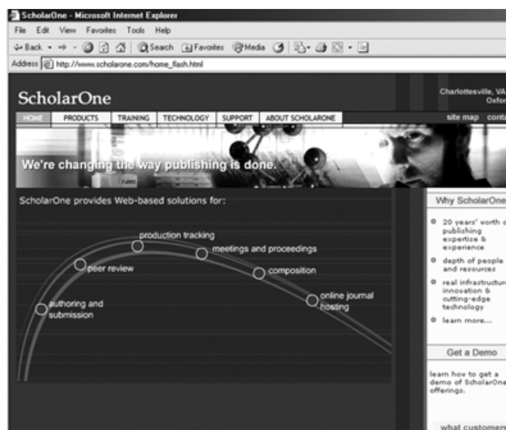

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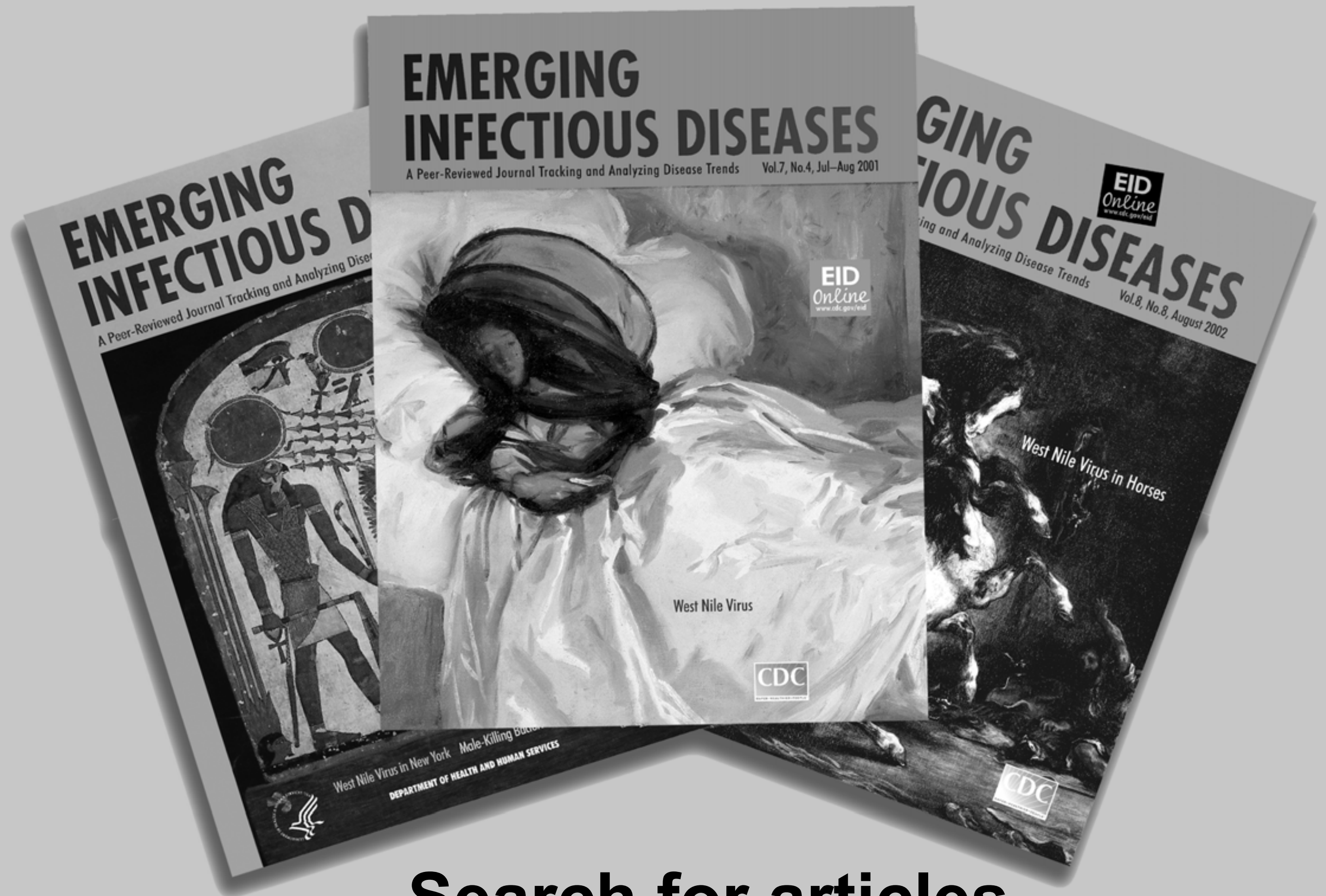

Search for articles on West Nile virus in past issues of

EMERGING INFECTIOUS DISEASES

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.

Manuscript Types

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.

Commentaries. 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.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. (500–1,000 words) of emerging infectious disease conferences may provide references to a full report of conference activities and should focus on the meeting's content rather than on individual conference participants.