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A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Vol.9, No.10, October 2003

CDC

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends pages 1197–1362

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Emerging Infectious Diseases
Emerging Infectious Diseases is published monthly by the National Center for Infectious Diseases, Centers for Disease Control and Prevention (CDC), 1600 Clifton Road, Mailstop D61, Atlanta, GA 30333, USA. Telephone 404-371-5329, fax 404-371-5449, email eideditor@cdc.gov.

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Jacques-Louis David (1748-1825).
Coronation of Empress Josephine by Napoleon I at Notre Dame de Paris, 2 December 1804 (1806-1807)
Oil on canvas, 6.1 m x 9.31 m. Photo: Peter Willi
Réunion des Musées Nationaux/Art Resource, NY. Chateaux de Versailles et de Trianon, Versailles, France

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Syndromic Surveillance and Bioterrorism-related Epidemics

James W. Buehler,* Ruth L. Berkelman,* David M. Hartley,† and Clarence J. Peters‡

To facilitate rapid detection of a future bioterrorist attack, an increasing number of public health departments are investing in new surveillance systems that target the early manifestations of bioterrorism-related disease. Whether this approach is likely to detect an epidemic sooner than reporting by alert clinicians remains unknown. The detection of a bioterrorism-related epidemic will depend on population characteristics, availability and use of health services, the nature of an attack, epidemiologic features of individual diseases, surveillance methods, and the capacity of health departments to respond to alerts. Predicting how these factors will combine in a bioterrorism attack may be impossible. Nevertheless, understanding their likely effect on epidemic detection should help define the usefulness of syndromic surveillance and identify approaches to increasing the likelihood that clinicians recognize and report an epidemic.

Because of heightened concerns about the possibility of bioterrorist attacks, public health agencies are testing new methods of surveillance intended to detect the early manifestations of illness that may occur during a bioterrorism-related epidemic. Broadly labeled "syndromic surveillance," these efforts encompass a spectrum of activities that include monitoring illness syndromes or events, such as medication purchases, that reflect the prodromes of bioterrorism-related diseases (1–9). The Centers for Disease Control and Prevention (CDC) estimates that, as of May 2003, health departments in the United States have initiated syndromic surveillance systems in approximately 100 sites throughout the country (T. Treadwell, CDC, pers. comm.). The goal of these systems is to enable earlier detection of epidemics and a more timely public health response, hours or days before disease clusters are recognized clinically, or before specific diagnoses are made and reported to public health authorities. Whether this goal is achievable remains unproved (4,5,10).

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Establishing a diagnosis is critical to the public health response to a bioterrorism-related epidemic, since the diagnosis will guide the use of vaccinations, medications, and other interventions. Absent a bioterrorism attack, predicting whether syndromic surveillance will trigger an investigation that yields a diagnosis before clinicians make and report a diagnosis is not possible. Our objective is to consider the mix of hypothetical factors that may affect the detection of epidemics attributable to CDC category A bioterrorism agents (11).

Establishing a Diagnosis

Two pathways to establishing a diagnosis are described by the scenarios below and in Figure 1, using a single, clandestine dissemination of an anthrax aerosol as an example.

Detection through Syndromic Surveillance

The early signs of inhalational anthrax include nonspecific symptoms that may persist for several days before the onset of more severe disease (12). Patients with prodromal illnesses seek outpatient care and are assigned nonspecific diagnoses such as "viral syndrome." Data on patients fitting various syndromic criteria are transferred to the health department and tested for aberrant trends. This process "flags" that a statistical detection threshold has been exceeded. Epidemiologists conclude that a preliminary investigation is warranted and collect blood for culture from several patients. Within 18 hours, one culture yields a presumptive diagnosis of anthrax, prompting a full-scale response.

Detection through Clinician Reporting

Some persons in whom inhalational anthrax develops will have short incubation periods and prodromes (12). Respiratory distress occurs in one such person, and he is hospitalized. Routine admission procedures include blood cultures. Within 18 hours, a presumptive diagnosis of anthrax is made. The patient's physician informs the local health department, prompting a full-scale response.

In practice, how a bioterrorism attack might be detected and diagnosed will probably be more complex.

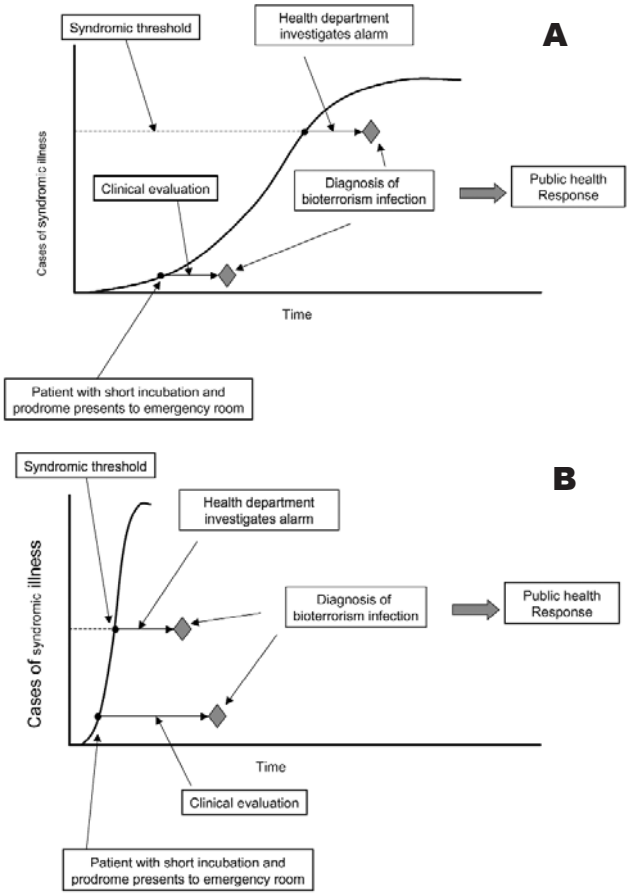

Figure 1. Number of cases of syndromic illness by time in a hypothetical bioterrorism attack and two pathways to establishing a diagnosis: syndromic surveillance coupled with public health investigation (upper pathway) and clinical and diagnostic evaluation of patients with short-incubation period disease (lower pathway). A, scenario favoring earlier detection by means of clinical evaluation. B, scenario favoring earlier detection by means of syndromic surveillance.

Published descriptions of 11 persons with inhalational anthrax in the United States in 2001 (13–19) provide some insight into this issue (Table 1 and Figure 2),[1] even though that epidemic was too small and geographically diffuse to be detectable by syndromic surveillance. For six patients with known dates of exposure, the median duration between exposure and symptom onset was 4 days (range 4–6 days). The median duration between onset and the initial healthcare visit was 3 days (20) (range 1–7 days), and the median duration between onset of symptoms and hospitalization was 4 days (range 3–7 days). Two of the 11 patients visited emergency departments and were sent home with diagnoses of gastroenteritis or viral syndrome 1 day before admission. In one patient, a blood culture obtained in the emergency room was read as positive for gram-positive bacilli the following day, which prompted recall of the patient. The culture was subsequently confirmed as positive for *Bacillus anthracis.* Two other patients were seen by primary care physicians and sent home with diagnoses of viral syndrome or bronchitis 2–3 days before admission, including one patient who was begun on empiric antibiotic therapy. For seven other patients, initial emergency room or hospital visits led directly to admission. In addition to the patient whose blood culture was obtained in an emergency room, seven others had not received prior antibiotic therapy, and *B. anthracis* was presumptively identified from blood within 24 hours of culture. One of these seven patients was the index patient, in whom *B. anthracis* was also recognized in cerebrospinal fluid within 7 hours of specimen collection. Three other patients had received antibiotics before blood cultures were taken (one as an outpatient and two at the time of hospital admission), requiring alternative diagnostic methods.

Despite the small number of patients, their experience offers four lessons for detecting an epidemic of inhalational anthrax. First, a key objective of syndromic surveillance is to detect early-stage disease, but fewer than half of these patients sought care before hospitalization was necessary, and the interval between such care and admission was relatively narrow (1–3 days). This finding suggests that syndromic surveillance data must be processed, analyzed, and acted upon quickly if such data are to provide a clue to diagnosis in advance of late-stage disease. Second, emergency room data are a common source for syndromic surveillance, but detecting an increase in visits coincident with hospital admission may not provide an early warning because the time needed to process surveillance data and investigate suspected cases would be at least as long as the time for admission blood cultures to be positive for *B. anthracis.* Blood cultures are likely to be routine for patients admitted with fever and severe respiratory illness, regardless of whether anthrax is considered as a diagnostic possibility, and *B. anthracis* grows readily in culture in the absence of prior antibiotic therapy, as observed in most of these patients. Thus, if emergency room data are to be useful in early detection of an anthrax epidemic, those data would need to be for visits that occur before hospital care is required—a pattern observed in only two patients. Third, the four patients who received early care and were discharged to their homes were assigned three different diagnoses, which suggests that syndromic surveillance systems must address the potential variability in how patients with the same infection may be diagnosed during the prodrome phase. Fourth, rapid diagnosis after hospitalization was possible only in those patients who had not received antibi-

[1]For interval calculations, if reported event dates were discrepant in different case reports, dates reported by Jernigan et al. (13) were used.

Table 1. Outcome of initial contact with health care for anthrax-related illness and timing of anthrax diagnosis, 11 patients with inhalational anthrax, 2001[a]

Outcome	No. of patients
Disposition after initial medical care	
Admitted to hospital	7
Discharged home from ER, subsequent hospital admission	2
Discharged home from outpatient provider, subsequent hospital admission	2
Total	11
Anthrax diagnosis	
Blood or CSF culture on hospital admission, presumptive diagnosis <24 h	7
Blood culture from preceding ER visit, patient recalled for admission	1
Prior antibiotic therapy; clinical suspicion of anthrax; specialized test required to establish diagnosis	3
Total	11

[a]ER, emergency room; CSF, cerebrospinal fluid.

otics before cultures were taken. This finding emphasizes the importance of judicious use of antibiotics in patients with nonspecific illness.

In addition to the specific attributes of individual bioterrorism agents, multiple considerations will shape the recognition of a bioterrorism-related epidemic. Five of these attributes follow.

Size

Syndromic surveillance would not detect outbreaks too small to trigger statistical alarms. Size would be affected by the virulence of the agent, its potential for person-to-person transmission, the extent and mode of agent dissemination, whether dissemination occurs in more than one time or place, and population vulnerability.

Population Dispersion

How persons change locations after an exposure will affect whether disease occurs in a concentrated or wide area, and thus whether clustering is apparent to clinicians or detectable through syndromic surveillance at specific sites.

Health Care

The more knowledgeable providers are about bioterrorism agents, the greater the likelihood of recognition. Routine diagnostic practices or access to reference laboratories may affect the timeliness of diagnosis for some diseases. Familiarity with reporting procedures would increase prompt reporting of suspected or diagnosed cases.

Syndromic Surveillance

Syndromic surveillance will be affected by the selection of data sources, timeliness of information management, definition of syndrome categories, selection of statistical detection thresholds, availability of resources for followup, recent experience with false alarms, and criteria for initiating investigations.

Season

A fifth key attribute is seasonality. An increase in illness associated with a bioterrorism attack may be more difficult to detect if it occurs during a seasonal upswing in naturally occurring disease.

Agent- and disease-specific attributes may be among the most important factors affecting detection and diagnosis (Table 2). The incubation period and its distribution in the population will affect the rate at which new cases

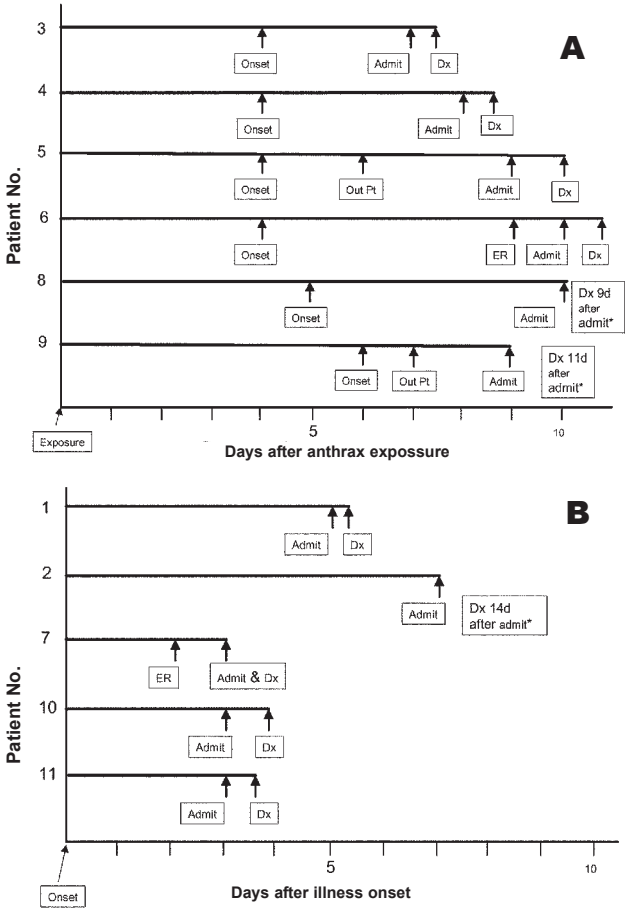

Figure 2. Timeline to presumptive anthrax diagnosis, 11 patients with inhalational anthrax, 2001, United States. Abbreviations: Dx, diagnosis; OutPt, outpatient visit followed by discharge home; ER, emergency room visit followed by discharge home. *Diagnosis delayed-initial blood cultures were negative in three patients who received antibiotic therapy before culture specimens were collected, requiring use of special diagnostic tests. For patients 1-10, case numbers correspond to those in report by Jernigan et al. (13); patient 11 reported by Barakat et al. (14). A, timeline begins with presumed date of anthrax exposure, available for six patients. B, timeline begins with day of illness onset for five patients without recognized date of exposure

Table 2. Characteristics of bioterrorism-related epidemics that affect detection through clinical recognition versus syndromic surveillance

Characteristics[a]	Clinical recognition[b]	Syndromic surveillance[c]
Duration and variability of incubation period	Broader distribution of incubation period increases likelihood that patient with short incubation-period disease would be diagnosed before a statistical threshold of syndromic cases is exceeded.	More narrow distribution of incubation period—leading to a steeper epidemic curve in the initial phase—increases likelihood that statistical threshold would be exceeded sooner.
Duration of nonspecific prodromal phase	Shorter prodrome increases likelihood of recognition or diagnosis at more severe or fulminant stage.	Longer prodrome increases likelihood that increase in syndromic manifestations would be detectable and that recognition of more severe stage (at which a diagnosis is more apt to be made) would be delayed.
Presence or absence of clinical sign that would heighten suspicion of diagnosis	Presence increases likelihood of earlier clinical recognition and diagnosis (e.g., mediastinal widening on chest x-ray in inhalational anthrax).	Absence decreases likelihood that diagnosis would be considered clinically, increasing opportunity for earlier detection by means of syndromic surveillance.
Likelihood of making diagnosis in the course of routine evaluation	If diagnosis is apt to be made in the course of a routine diagnostic evaluation (not dependent on clinical suspicion of specific bioterrorism infection), early diagnosis through clinical care is likely.	If diagnosis is dependent on the use of a special test that is unlikely to be ordered in the absence of clinical suspicion of diagnosis, then diagnosis in clinical care may be delayed, increasing the opportunity for early detection through syndromic surveillance.

[a]Infection or disease attributes that may affect detection of an epidemic.
[b]Increases likelihood of initial detection through routine clinical care and reporting.
[c]Increases likelihood of initial detection through syndromic surveillance.

develop (21) and thus how quickly an alarm threshold is exceeded or whether clinicians recognize a temporal and geographic cluster. If a disease has a short prodrome, the chance is increased that a patient would be hospitalized and a definitive evaluation initiated before an increase in cases triggered a surveillance alarm. Alternatively, if a disease has a relatively long prodrome, chances are greater that prediagnostic events (e.g., purchase of medications or use of outpatient care for nonspecific complaints) would accrue to levels that exceed syndromic surveillance thresholds, before definitive diagnostic evaluations are completed among patients with more severe disease. Arousing clinical suspicion for a particular diagnosis will depend on the specificity of both the early and late stages of illness as well as the presence or absence of a typical feature that should alert clinicians to the diagnosis, such as mediastinal widening in inhalational anthrax (12). If a routinely performed test is apt to be diagnostic in a short time (e.g., the blood culture in anthrax), a rapid diagnosis is likely, even in the absence of clinical suspicion. If routine tests are unlikely to yield a rapid diagnosis (e.g., the blood culture for the cause of tularemia, *Francisella tularensis* [22]), or if the diagnosis requires a special test (e.g., the hemorrhagic fever viruses [23]), a diagnosis may be delayed if not immediately considered.

The public health benefit resulting from early detection of an epidemic is likely to vary by disease. If a disease has a relatively wide distribution of potential onsets, early recognition provides greater opportunity to administer prophylaxis to exposed persons. For example, based on data from the Sverdlovsk incident (24), Brookmeyer and Blades estimated that use of antibiotic prophylaxis during the 2001 anthrax outbreak prevented nine cases of inhalational disease among exposed persons (25). If the incubation period of a disease has a relatively narrow distribution, early recognition may offer little opportunity for postexposure prophylaxis, although a potential benefit would remain for alerting healthcare providers and informing their care of others with similar symptoms. This pattern of illness is apt to result from exposure to an *F. tularensis* aerosol, which would likely result in an explosive epidemic with an abrupt onset and limited duration (22).

Detecting Specific Bioterrorism Epidemics and Agents

The attributes of the CDC category A bioterrorism agents that affect their detection, as well as the benefits of early detection, are summarized below, on the basis of potential bioterrorism-related epidemic profiles developed by experts (12,22,23,26–28). These profiles reflect current knowledge of these diseases; their epidemiology might differ if novel modes of dissemination or preparation were employed. Each disease has attributes that could increase or decrease the likelihood of early outbreak recognition through either clinical diagnosis or syndromic surveillance.

Inhalational Anthrax

The distribution of the incubation period for inhalational anthrax can be relatively broad as observed in Sverdlovsk (2–43 days); most cases occur within 1–2 weeks after exposure (24). In the 2001 U.S. outbreak, the distribution of incubation periods was more limited, 4–6 days, although later-onset cases may have been averted by antibiotic prophylaxis (25). The nonspecific prodrome for anthrax may last from several hours to several days. Taken together, these data suggest that the initial slope of an epidemic curve may be comparatively gradual during the first

week, leading to slower recognition through syndromic surveillance than for other infections caused by bioterrorist agents with pulmonary manifestations, such as tularemia or pneumonic plague (22,28). In contrast, mediastinal widening on chest x-ray or computed tomographic scan or Gram stain of cerebrospinal or pleural fluid should lead an alert and knowledgeable physician to consider the diagnosis of anthrax, even though these tests may not be conducted until relatively late in the clinical course. *B. anthracis* is likely to be detected quickly in cultures, favoring clinical recognition. Retrospective analysis of data from 2001 showed that inhalational anthrax can be distinguished from influenzalike illness or community-acquired pneumonia by using an algorithm that combines clinical and laboratory findings (20), although the practical utility of this approach is untested. In addition to permitting antibiotic use among ill persons, early recognition would enable postexposure antibiotic prophylaxis (12,25).

Tularemia

The typical incubation period for tularemia is relatively narrow after a person is exposed to aerosolized *F. tularensis,* with abrupt onset of nonspecific febrile illness, with or without respiratory symptoms, in 3–5 days (range 1–14 days), followed by rapid progression to life-threatening pneumonitis (22). This relatively narrow incubation period for most patients and rapid progression to severe disease would lead to a rapid increase in cases after a large and acute exposure. Finding a number of such cases in a short interval should trigger both syndromic surveillance alarms and clinical suspicion. *F. tularensis* is a slow-growing and fastidious organism and may take up to 5 days after inoculation to be detectable, if it is detected at all, in a routinely processed blood culture. The use of special laboratory techniques may be required, delaying the likelihood of detection in the absence of clinical suspicion. After an epidemic is recognized, specific antibiotic therapy is recommended for exposed persons in whom a febrile illness develops (22).

Pneumonic Plague

Exposure to aerosolized *Yersinia pestis* results in pneumonic plague, which has a typical incubation period of 2 to 4 days (range 1–6 days). The disease has a relatively short prodrome, followed by rapidly progressive pneumonia (28), which would lead to a rapid increase in cases at the onset of an epidemic. Standard clinical laboratory findings are nonspecific, which alone might not prompt clinical suspicion, but microscopic examination of a sputum smear may show characteristic findings, which should prompt consideration of the diagnosis. Cultures of blood or sputum are apt to show growth within 24 to 48 hours, but routine procedures may misidentify *Y. pestis* unless the diagnosis is suspected and special attention is given to specimen processing. Confirming the diagnosis depends on special tests available through reference laboratories. Treatment the first day of symptoms is generally considered necessary to prevent death in pneumonic plague, so early recognition of an aerosol plague attack would enable life-saving use of antibiotics in febrile patients and prophylaxis of contacts (28).

Botulism

Foodborne botulism typically has a relatively narrow incubation period (12–72 hours), which may vary from 2 hours to 8 days, depending on the inoculum. For the three known cases of inhalational botulism attributed to a relatively low exposure to aerosolized toxin, the incubation period was approximately 72 hours (26). The characteristic clinical picture of descending paralysis should prompt consideration of botulism, and this unique pattern among bioterrorism agents lends itself to a specific syndrome category. However, the illness may be misdiagnosed, as observed in a large foodborne outbreak of botulism in 1985; 28 persons who had eaten at a particular restaurant and in whom botulism had developed were assigned other diagnoses before the geographically dispersed outbreak was recognized and publicized in the media (26,29). Symptoms of inhalational botulism, with choking, dysphagia, and dysarthria dominating the clinical picture, may differ from those associated with ingestion of toxin and complicate recognition of the disease. Specialized testing for botulinum toxin is available at a limited number of state laboratories and CDC. Postexposure prophylaxis is limited by the scarcity of, and potential for, allergic reactions to botulinum antitoxin, leading to recommendations that exposed persons be observed carefully for early signs of botulism, which should prompt antitoxin use (26). Antitoxin should be given as early as possible, another fact that highlights the importance of early detection. Depending on the level of exposure and the geographic dispersion of affected persons, syndromic surveillance for characteristic neurologic symptoms could aid outbreak detection, or the occurrence of an epidemic might be obvious to clinicians.

Smallpox

The incubation period of smallpox is usually 12–14 days but may range from 7 to 17 days. The early symptomatic phase includes a severe febrile illness and appearance of a nonspecific macular rash over a 2- to 4-day period, followed by evolution to a vesicular and then pustular rash over the next 4 to 5 days (27). Thus, the initial phase of smallpox may lend itself to detection through surveillance of a febrile rash illness syndrome. Once smallpox is suspected, the virus can be rapidly detected by electron

microscopic examination of vesicular or pustular fluid, if laboratory resources for electron microscopy are available, or by polymerase chain reaction, if the necessary primers are available. Contacts can be protected by vaccination up to 4 days after exposure. Discourse is substantial about the relative merits of pre-event versus postevent vaccination (27,30–33). Syndromic surveillance may show an increase in febrile rash illness, although once the characteristic rash appears, the diagnosis should be quickly established.

Viral Hemorrhagic Fevers

This category includes multiple infectious agents that range from having a relatively broad to narrow incubation period (e.g., Ebola, 2–21 days; yellow fever 3–6 days). These diseases present with nonspecific prodromes that may have an insidious or abrupt onset. In severe cases, the prodrome is followed by hypotension, shock, central nervous system dysfunction, and a bleeding diathesis. The differential diagnosis includes a variety of viral and bacterial diseases. Establishing the diagnosis depends on clinical suspicion and the results of specific tests that must be requested from CDC or the U. S. Army Medical Research Institute of Infectious Diseases. The value of postexposure prophylaxis with antiviral medications is uncertain, and (with the exception of yellow fever, for which a vaccine is available) response measures are limited to isolation and observation of exposed persons, treatment with ribavarin (if the virus is one that responds to that antiviral drug), and careful attention to infection control measures (23). Patients seen with symptoms during the prodromal phase may not clearly fit into a single syndrome category, but syndromic surveillance focused on the early signs of a febrile bleeding disorder would be more specific.

One of the biggest concerns about syndromic surveillance is its potentially low specificity, resulting in use of resources to investigate false alarms (6,10). Specificity for distinguishing bioterrorism-related epidemics from more ordinary illness may be low because the early symptoms of bioterrorism-related illness overlap with those of many common infections. Specificity for distinguishing any type of outbreak from random variations in illness trends may be low if statistical detection thresholds are reduced to enhance sensitivity and timeliness. The likelihood that a given alarm represents a bioterrorism event will be low, assuming that probability of such an event is low in a given locality. Approaches used to increase specificity include requiring that aberrant trends be sustained for at least 2 days or that aberrant trends be detected in multiple systems (2). Another approach to enhancing specificity would be to focus surveillance on the severe phases of disease, since the category A bioterrorism infections are more likely than many common infections to progress to life-threatening illness. For those diseases that are likely to progress rapidly, such as pneumonic plague, syndromic detection of severe disease (e.g., through emergency room visits, hospital admissions, or deaths) may be more feasible than detection aimed at early indicators before care is sought (e.g., purchases of over-the-counter medications) or when illness is less severe (e.g., primary care visits). Whether detection of syndromic late-stage disease offers an advantage over detection through clinical evaluation will depend on the attributes of the infections and diagnostic resources, as described above.

Predicting how the mix of relevant factors would combine in a given situation to affect the recognition of a bioterrorism-related epidemic is difficult, although mathematical models may provide further insight (5). The most important factors affecting early detection are likely to be the rate of accrual of new cases at the outset of an epidemic, geographic clustering, the selection of syndromic surveillance methods, and the likelihood of making a diagnosis quickly in clinical practice.

Ongoing efforts to strengthen the public health infrastructure (34,35) and to educate healthcare providers about bioterrorism diseases and reporting procedures should strengthen the ability to recognize bioterrorism outbreaks. For example, in New Jersey in 2001, reporting of two early cases of cutaneous anthrax was delayed until publicity about other anthrax cases prompted physicians to consider the diagnosis and notify the health department, suggesting that opportunities for earlier use of postexposure prophylaxis were missed (36). In addition, while the importance of new diagnostic tools, including rapid tests, should be emphasized (37), the essential role of existing diagnostic techniques should not be overlooked. Clinical suspicion is critical, and a key prompt for arousing clinical suspicion may be the microscopic examination of a routinely collected specimen, as occurred in the index case of the 2001 anthrax outbreak, when a Gram stain of the cerebrospinal fluid led to the diagnosis (15). However, as recently highlighted by the Institute of Medicine, the use of basic diagnostic tests has decreased because of efforts to reduce the costs of care, the increasing use of empiric broad-spectrum antibiotic therapy, and federal laboratory regulations, such as the Clinical Laboratory Improvement Amendments of 1988, which have discouraged laboratory evaluation in some clinical settings (38).

While we have focused on the role of syndromic surveillance in detecting a bioterrorism-related epidemic, other uses of syndromic surveillance include detecting naturally occurring epidemics, providing reassurance that epidemics are not occurring when threats or rumors arise, and tracking bioterrorism-related epidemics regardless of the mode of detection (4,6,10). Syndromic surveillance is intended to enhance, rather than replace, traditional approaches to epidemic detection. Evaluation of syn-

dromic surveillance to consider the spectrum of potential uses is essential. A certain level of false alarms, as the result of either syndromic surveillance or calls from clinicians, will be necessary to ensure that opportunities for detection are not missed. Efforts to enhance the predictive value of syndromic surveillance will be offset by costs in timeliness and sensitivity, and defining the right balance in practice, particularly in the absence of an accurate assessment of bioterrorism risk, will be essential.

Two committees of the National Academies have recommended more careful evaluation of the usefulness of syndromic surveillance before it is more widely implemented (5,38). Because the epidemiologic characteristics of different bioterrorism agents may vary in ways that affect the detection of epidemics, these evaluations should address the epidemiology of specific bioterrorism agents. Efforts to detect bioterrorism epidemics at an early stage should not only address the development of innovative new surveillance mechanisms but also strengthen resources for diagnosis and enhance relationships between clinicians and public health agencies—relationships that will ensure that clinicians notify public health authorities if they suspect or diagnose a possible bioterrorism-related disease.

Acknowledgments

We wish to acknowledge the contributions of the anonymous reviewers.

Drs. Buehler and Berkelman were supported in part by a grant from the O. Wayne Rollins Foundation.

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Illness in Intensive Care Staff after Brief Exposure to Severe Acute Respiratory Syndrome

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Severe acute respiratory syndrome (SARS) is a threat to healthcare workers. After a brief, unexpected exposure to a patient with SARS, 69 intensive-care staff at risk for SARS were interviewed to evaluate risk factors. SARS developed in seven healthcare workers a median of 5 days (range 3–8) after last exposure. SARS developed in 6 of 31 persons who entered the patient's room, including 3 who were present in the room >4 hours. SARS occurred in three of five persons present during the endotracheal intubation, including one who wore gloves, gown, and N-95 mask. The syndrome also occurred in one person with no apparent direct exposure to the index patient. In most, but not all cases, developing SARS was associated with factors typical of droplet transmission. Providing appropriate quarantine and preventing illness in healthcare providers substantially affects delivery of health care.

Severe acute respiratory syndrome (SARS) is a disease that consists of fever and respiratory symptoms that can progress to respiratory failure and death (1). SARS is most likely to develop in healthcare workers and household or family contacts of infected persons (2–4). Unprotected exposure to SARS in hospitals has several potential consequences, which include the following: illness in persons and healthcare workers; transmission of SARS from ill healthcare workers and patients to visitors and household contacts; and reduced ability of the healthcare system to deliver care because of illness in or quarantine of healthcare workers. In addition, the psychological impact of isolation and quarantine can be substantial (5). As a result, understanding factors associated with SARS transmission after exposure to SARS patients is important and would assist with formulating appropriate quarantine procedures. We describe our experience with a large number of healthcare workers who were exposed to a patient in an intensive-care unit (ICU) with undiagnosed SARS.

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Index Patient

On March 23, 2003, a 74-year-old immunocompromised man was transferred to our ICU from a hospital where the original cluster of Toronto's SARS cases occurred (2). The patient originally had signs and symptoms consistent with a presumptive diagnosis of community-acquired pneumonia. Before transfer, SARS was excluded from the differential diagnosis because the patient had not traveled, had never left the emergency department of the referring hospital, and had only had a single recent outpatient visit to an area of the original hospital in which SARS had not been identified. Upon arrival in our ICU, the patient was placed in precautions for methicillin-resistant *Staphylococcus aureus* (MRSA) pending admission screening results (6). Therapy with broad-spectrum antimicrobial drugs was initiated. Humidified high-flow oxygen was administered for the first 5 h, noninvasive positive pressure ventilation by oronasal mask for the next 18.25 h, and invasive mechanical ventilation for the subsequent time (7.5 h). Endotracheal intubation required fiber-optic placement. That the extent of the outbreak at the referring institution was larger than originally appreciated became apparent at this time; therefore, the patient was transferred to another facility for placement in negative pressure isolation for possible exposure to SARS. Subsequently, his family members became ill, and the SARS-associated coronavirus was identified in the patient's respiratory secretions (polymerase chain reaction testing of bronchoalveolar lavage confirmed the diagnosis of SARS).

Quarantine

Once the risk for SARS was identified, all patients in the ICU were considered to have been potentially exposed. To prevent spread of SARS, we closed the ICU to admissions and discharges and implemented strict respiratory and contact precautions for all remaining patients. We quarantined 69 healthcare workers who were considered to be at high risk for developing SARS. On the basis of our

understanding of disease transmission, we arbitrarily decided that persons at high risk included anyone who had entered the index patient's room or who had been in the ICU for >4 hours during the patient's 30.75-h stay.

Methods

After research ethics board approval and informed consent, two researchers used a structured questionnaire to interview quarantined healthcare workers. The questionnaire elicited demographic information, details about health, and information about exposure to the index patient. Time of exposure was categorized as follows: <1 min, 1–10 min, 11–30 min, 31–60 min, 1–4 h, or >4 h. Exposure proximity, procedures performed, and infection-control precautions were documented. Each healthcare worker was asked about symptoms suggestive of SARS that developed during or after the quarantine period.

For healthcare workers in whom suspected or probable SARS developed, additional data were collected about the nature and course of their illness. Suspected and probable SARS were defined according to the definitions issued by the World Health Organization (WHO) (7). Symptoms of suspected SARS were a fever >38°C, respiratory symptoms, and an epidemiologic link with a SARS patient; all quarantined healthcare workers were considered to have an epidemiologic link on the basis of contact with the index patient. Probable SARS was defined as suspected SARS with radiographic lung infiltrates.

Statistics

All data were entered into an Access (Microsoft Corp., Redman, WA) database by using double data entry technique and analyzed by using SAS version 8.0 (SAS Institute, Inc., Cary, NC). For comparisons of characteristics of healthcare workers with SARS to those of healthcare workers without SARS, we used the two-sample t test for normally distributed variables, Wilcoxon rank sum test for ordinal and skewed continuous variables, and Fisher exact test for categorical variables. Two-sided tests were used for all comparisons. A p value of <0.05 was considered to be statistically significant. Classification and regression tree methods were used to identify predictors of developing SARS (8). The healthcare workers were divided into two groups by examining all possible cutpoints of all predictor variables to find the cutpoint of a predictor variable that resulted in the largest difference in the probabilities of developing SARS between the two resulting subgroups. This procedure was performed repeatedly for each resulting subgroup until all members of the subgroup had the same SARS status or the subgroup was too small to warrant further splitting.

Results

Of the 69 quarantined patients, 63 were interviewed. Five declined, and one could not be contacted. SARS did not develop in healthcare workers who were not quarantined and patients who had been in the unit at the time of the exposure.

SARS Development

SARS developed in 7 of the 69 quarantined healthcare workers (6 probable, 1 suspected; Table 1). One healthcare worker had a history of type II diabetes mellitus; all other healthcare workers were previously healthy. The median time from exposure to the index patient to onset of symptoms was 5 days (range 3–8 days). All probable case-patients were hospitalized and required oxygen but did not require ICU care. Treatment with levofloxacin (500 mg once a day for 7 days) and ribavirin (2,000–2,200 mg loading dose followed by 1,200 mg every 6 h for 4 days and subsequent tapering off) was administered to all admitted case-patients, and all but one received systemic corticosteroids (1 mg/kg prednisone or equivalent once a day for 5 days with subsequent tapering off). The median hospital stay was 19.5 days (range 13–25 days). All case-patients were discharged. However, 28–32 days after discharge, all reported continued dyspnea with exercise.

Room Visitation

Thirty-one healthcare workers had entered the index patient's room; SARS developed in 6 (19%). The contact characteristics and infection control precautions used by the healthcare workers who entered the patient's room are shown in Table 2. All six healthcare workers in whom SARS developed and who entered the patient's room reported being present >11 min; three were in the room for >4 hours. SARS attack rates were higher among healthcare workers who spent more time in the index patient's room; in addition, a dose-response effect occurred between duration of exposure and risk of developing SARS (Table 3).

Contact with Index Patient

All six healthcare workers with SARS who entered the index patient's room also touched the patient, and all reported performing a procedure that involved contact with the patient's mucous membranes or respiratory secretions (Table 2). Three of the six healthcare workers reported wearing gloves during this contact. In contrast, of the 13 healthcare workers without SARS, 12 (92%) used gloves when touching the patient (odds ratio [OR] 0.08, 95% confidence interval [CI] 0.01 to 1.11, p=0.07). Selected contact characteristics predictive of the development of SARS in healthcare workers who entered the patient's room appear in the Figure.

Table 1. Description of healthcare workers in whom severe acute respiratory syndrome developed

Patients	Occupation	Duration of exposure to index patient	Precautions	Special considerations
Patient 1	Registered nurse	22 h	Gown, gloves, surgical mask[b]	• Present during intubation of airway • Performed all primary nursing activities on 2 shifts
Patient 2	ICU resident	31–60 min	N-95 mask, gown, gloves	• Performed difficult intubation of airway
Patient 3	Registered nurse	None	Not applicable	• Assigned to patient 3 rooms down hall from index patient
Patient 4	Registered nurse	31–60 min	Gown, gloves, surgical mask	• Assisted primary nurse with bathing of index patient
Patient 5	Anesthetist	11–30 min	Gown, gloves, surgical mask	• Performed difficult intubation of airway
Patient 6	Respiratory therapist	4 h	None	• Instituted NPPV • Inserted arterial line
Patient 7[c]	Respiratory therapist	6 h	Gown, gloves[b]	• Instituted NPPV • Frequently manipulated oxygen mask

[a]ICU, intensive-care unit; NPPV, noninvasive positive-pressure ventilation.
[b]Denotes precautions that were taken by the healthcare worker sometimes but not always during exposure.
[c]Patient 7 has been classified as a suspected case, as she did not have radiographic lung infiltrates.

SARS developed in three of the five persons present during the endotracheal intubation of the patient. During this procedure, the patient's respiratory secretions were splashed onto the uncovered cheek of one of the healthcare workers. No other healthcare worker reported direct skin exposure to the patient's bodily secretions at any time during his admission. Two of the three persons in whom SARS developed after the endotracheal intubation wore a gown, surgical mask, and gloves; one healthcare worker wore a gown, gloves, and N-95 mask. Of the two healthcare workers present during endotracheal intubation in whom SARS did not develop, one was a postgraduate medical trainee who assisted with manual ventilation (bag-valve-mask ventilation using a Laerdal bag) and was positioned to the side of the patient rather than directly over the patient's head. This healthcare worker wore gown, gloves, and surgical mask during the procedure. The second worker was a respiratory therapist who helped prepare the necessary equipment while wearing gown, gloves, and an N-95 mask.

Of the healthcare workers who entered the index patient's room, 22 were present at some time during the administration of noninvasive positive-pressure ventilation (NPPV), and SARS developed in 4 (18%). Each of these 4 healthcare workers, but only 1 of the 18 healthcare workers who remained well, reported being present in the room for >31 minutes during the administration of NPPV (OR 105, 95% CI 3 to 3,035, $p \leq 0.001$). The one worker in whom SARS did not develop despite being present during NPPV therapy for >31 minutes wore a surgical mask, gown, and gloves. One of the 4 healthcare workers in whom SARS developed and 4 of the 18 healthcare workers

Table 2. Contact characteristics and infection control precautions for 31 healthcare workers who entered index patient's room[a]

Exposure type	No. healthcare workers with exposure	No. (%) exposed healthcare workers with SARS
Entry into room	31	6 (19)
Contact duration for those entering the room		
≤10 min	11	0
11–30 min	8	1 (12.5)
31 min to 4 h	8	2 (25)
≥4 h	4	3 (75)
Nature of contact		
Touched patient	19	6 (32)
Contact with mucous membranes	10	4 (40)
Performed procedure involving contact with mucous membranes or respiratory secretions	15	6 (40)
Present during NPPV	22	4 (18)
Performed or assisted intubation	5	3 (60)
Infection control precautions used during exposure		
Always wore at least:		
Gloves	15	3 (20)
Gown and gloves	15	3 (20)
Any mask (N-95 or surgical mask)	13	3 (23)
Gown, gloves, and N-95 mask	6	1 (17)
Gown, gloves, and surgical mask	6	2 (30)
Gown, gloves, and any mask	12	3 (25)
No precautions	8	1 (12.5)

[a]NPPV, noninvasive positive-pressure ventilation.

Table 3. Development of severe acute respiratory syndrome (SARS) in healthcare workers, depending on time spent in index patient's room (N=31)[a]

Time spent in index patient's room	No. (%) healthcare workers with specified exposure with SARS	No. (%) healthcare workers without specified exposure with SARS	Odds of developing SARS after specified exposure	95% CI for OR	p value
≤10 min	0/11	6/20 (30)	0.097[b]	(0.005 to 1.91)[b]	0.07
≥31 min	5/12 (42)	1/19 (5)	12.9	(1.27 to 131)	0.022
≥4 h	3/4 (75)	3/27 (11)	24.0	(1.85 to 311)	0.016

[a]CI, confidence interval; OR, odds ratio.
[b]These logit estimators use a correction of 0.5 in every cell of the table that contains a zero.

who remained well wore an N-95 mask during NPPV administration.

No Room Visitation

SARS developed in one quarantined healthcare worker (a nurse) who had not entered the index patient's room; the disease did not occur in any other healthcare workers who had not touched or had close contact with the index patient. The nurse was present in the ICU for 18.75 h (two shifts) during the patient's admission. Of note, after the endotracheal intubation of the index patient, the physician who performed this procedure entered the room where the nurse was caring for another patient. Neither the nurse nor the physician recalled direct contact, and they were certain that the physician had changed gloves and gown before room entry. This nurse had no other epidemiologic risk to explain the development of SARS.

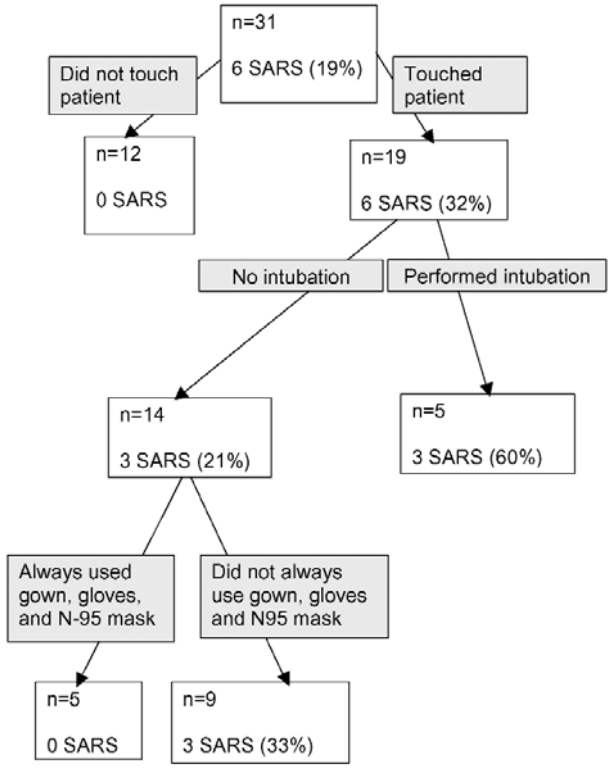

Figure. Regression tree describing selected contact characteristics in healthcare workers who entered the index patient's room. Does not include results for one healthcare worker who had no history of entering the index patient's room but nevertheless acquired severe acute respiratory syndrome.

Other Observations

One healthcare worker spent >4 hours with the index patient; however, SARS did not develop in this worker. This worker wore an N-95 mask, gloves, and gown during exposure and was not present during the endotracheal intubation or during the administration of NPPV. SARS did develop in another healthcare worker who performed the endotracheal intubation while wearing an N-95 mask, gown, and gloves.

Discussion

Our results suggest that proximity and duration of contact to a patient with SARS are associated with risk for viral transmission, an observation suggested by others (2–4). In addition, certain procedures, such as endotracheal intubation, pose increased risk. These findings may be predictable given that SARS is thought to spread primarily by large droplets (9).

Three of the six persons in whom SARS developed after entering the index patient's room may not have adhered to standard MRSA precautions in that they performed procedures which involved contact with mucous membranes without wearing gloves. Furthermore, we were unable to determine if hand washing impacted SARS transmission, as this information was not collected.

During our study, we made two important observations. First, SARS developed in one healthcare worker despite the fact that the worker wore an N-95 mask, gown, and gloves. Second, SARS developed in another healthcare worker who had no identified contact with the index patient or with any other persons known to have SARS. In the case of the first healthcare worker, the absence of eye protection may have contributed to disease transmission. In addition, although this person wore an N-95 mask while in the patient's room, he had not been fit-tested for this mask; however, fit-testing should not be necessary if the SARS-associated coronavirus is spread by large droplets (6). As a result of this and similar episodes of SARS transmission in the Toronto area (10), the province of Ontario has now made specific recommendations for

healthcare workers performing intubation that involve increased protection (available from: URL: www.sars.medtau.org) (11), and protective eye wear is currently mandated for patient encounters. In the second case, transmission could have occurred in a number of possible routes. The nurse may have come within sufficient range of the SARS patient to be exposed to large droplets. Recent reports indicate that the virus may survive for several hours on fomites or in body secretions (12) and raise the possibility of transmission by indirect contact with contaminated objects or of inadvertent carriage and spread by another healthcare worker. Fecal transmission is unlikely as the patient did not have a bowel movement during his stay. True airborne spread may also have occurred. Although evidence does not support this route of transmission for the SARS-associated coronavirus, existing literature suggests that other coronaviruses may be spread by an airborne route in certain circumstances (13).

Given our lack of knowledge about the transmissibility of SARS at the time this exposure occurred, we made a conservative decision to quarantine for 10 days all persons who were in the unit for at least 4 h or who had a history of entry into the affected patient's room. In addition, we closed the ICU to admissions and discharges for a 10-day period, markedly affecting our institution's ability to deliver health care. In fact, during the Toronto outbreak, several of the city's ICUs were closed as a result of quarantine and illness in staff with similar consequences (14); by infecting healthcare workers, SARS has an impact on the health of an entire community. A less aggressive quarantine approach may have been as effective in controlling transmission and allowed more staff to be available for work. For instance, only persons who have had direct contact with the patient (i.e., entered the patient's room) could have been quarantined. If we had taken this approach, the quarantine would have excluded six persons with SARS from the workplace but only removed 25 of the 62 persons who remained well. However, this approach would have missed one healthcare worker in whom SARS developed. Another approach might be to monitor staff closely for SARS-related symptoms while they continue their usual activities and quarantine only those in whom symptoms occur. This approach would require evidence that SARS cannot be transmitted before symptom onset, confidence in the facility's ability to identify symptomatic staff, and reliability of healthcare workers in reporting symptoms. We think that our quarantine approach prevented secondary spread of illness to other persons who may have come in contact with the workers in whom SARS developed.

Our study involved a small number of cases, and definitive conclusions cannot be drawn from a report of this size. For example, although SARS developed in our staff within the 10-day quarantine period, others have demonstrated that the time period from infection to onset of symptoms may be >10 days (15). One of the strengths of our study is that the exposure occurred during a defined period in a contained unit, and as such, there is less potential for confounding caused by the exposure of healthcare workers to multiple SARS patients.

Our observations emphasize the consequences of missing the diagnosis of SARS for even a relatively brief period. In our experience, we would make the following recommendations. First, the possibility of unexpected exposure of healthcare workers to patients with SARS should be anticipated, and once such exposure is recognized, those deemed to be at risk for SARS transmission should be promptly quarantined. Second, vigilant surveillance for symptoms of SARS must be maintained by all healthcare workers who work in institutions with SARS patients; SARS may develop in healthcare workers even when they do not have direct exposure to patients with SARS. In addition, protocols for managing patients with SARS should include not only contact and respiratory precautions but also procedures that minimize patient contact since duration and proximity of contact increase the risk for transmission of SARS. Finally, additional precautions should be taken when performing high-risk procedures, such as endotracheal intubation (11).

Though many of the healthcare workers in our ICU were exposed to the patient with SARS, our experience suggests that the greatest risk for SARS transmission occurs in those healthcare workers with prolonged exposure or direct physical contact with the patient. Use of gowns, gloves, and masks as barriers appears to reduce the risk for SARS transmission in most but not all situations. Additional information will be needed to determine if modes of transmission beyond droplet spread are important. We think this information will be helpful to institutions dealing with similar exposures to patients with SARS and developing quarantine protocols.

Acknowledgments

We thank Patrick Cheng, Margaret McArthur, and Agron Plebneshi for their assistance with data entry; Ron Heslegrave for his advice regarding the consent form; Farida Hasin-Shakoor for administrative assistance; and Allan S. Detsky and Arthur S. Slutsky for critically reading the manuscript.

Two of our investigators (K.G., R.S.) were supported in part by a grant from the Ontario Ministry of Health and Long-Term Care.

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Superantigens and Streptococcal Toxic Shock Syndrome

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Superantigens produced by *Streptococcus pyogenes* have been implicated with streptococcal toxic shock syndrome (STSS). We analyzed 19 acute-phase serum samples for mitogenic activity from patients with severe streptococcal disease. The serum samples from two patients in the acute phase of STSS showed strong proliferative activity. Streptococcal mitogenic exotoxin (SME) Z-1 and streptococcal pyrogenic exotoxin (SPE)-J were identified in one patient with peritonitis who recovered after 2 weeks in intensive care. SMEZ-16 was found in a second patient who died on the day of admission. Sequential serum samples taken on day 3 after admission from patient 1 showed clearance of mitogenic activity but absence of neutralizing anti-SMEZ antibodies. Serum samples taken on day 9 from this patient showed evidence of seroconversion with high levels of anti-SMEZ antibodies that neutralized SMEZ-1 and 12 other SMEZ-variants. These results imply that a high level of SMEZ production by group A streptococcus is a causative event in the onset and subsequent severity of STSS.

Since the 1980s, a marked increase has occurred in highly invasive group A streptococcal (GAS) infections, in particular streptococcal toxic shock syndrome (STSS) associated with necrotizing fasciitis or myositis (1–4). The classical case definition for STSS is similar to staphylococcal toxic shock, caused by *Staphylococcus aureus,* but the outcome is more serious in STSS, with a reported death rate of 30% to 70% (2,5,6).

The multiorgan involvement in STSS suggests that a toxin produced by GAS might be involved in pathogenesis. Prime candidates are the streptococcal superantigens (SAgs), a family of highly mitogenic proteins secreted individually or in certain combinations by many *Streptococcus pyogenes* strains (7–10), although other virulence factors, such as streptolysin O and various cell wall antigens can also cause toxic shock (11). Superantigens simultaneously bind to major histocompatibility complex class II molecules and T-cell receptor molecules bearing a particular V-β region. This binding results in the activation of a large proportion of antigen-presenting cells and T cells, with subsequent release of high systemic levels of cytokines (12–15).

Several lines of evidence support the hypothesis of SAg involvement in STSS. Toxic shock syndrome (TSS) toxin, produced by *S. aureus*, has been associated with most menstrual TSS cases (7). TSS toxin is a typical SAg that is functionally and structurally related to the staphylococcal and streptococcal SAgs (16). Moreover, animal models have shown that TSS toxin and other SAgs induce TSS-like symptoms in rabbits and rodents (17,18). The lack of neutralizing anti-SAg antibodies appears to be a key risk factor for the development of staphylococcal and streptococcal toxic shock (19,20).

The major cytokines released from antigen-presenting cells and T cells after activation by SAgs are tumor necrosis factor alpha (TNF-α), tumor necrosis factor beta (TNF-β), interleukin (IL)-1, and IL-2 (11–14). TNF-α is the prime mediator of shock; anti–TNF-α has been shown to inhibit the progression of SAg-driven shock in mice and baboons (17,18,21).

In contrast to TSS toxin and staphylococcal TSS, the association of individual streptococcal SAgs to STSS is much less understood. Several studies described the potential involvement of streptococcal pyrogenic exotoxin (SPE) A in invasive streptococcal disease (2,19,22,23), while others reported an association with SPE-C (24,25). In addition, some cases of STSS are not associated with SPE-A or SPE-C (26). Notably, these studies were performed without knowledge of other streptococcal SAgs that are now known to exist.

Superantigen activity found in acute-phase serum samples from streptococcal disease patients has been reported. (In this article, the term "acute-phase serum" refers to serum taken on the day of admission). Sriskandan et al. published a study of seven patients with severe streptococcal infections: SPE-A was detected in serum samples from four patients (27). Recently, Norby-Teglund and Berdal reported a strong proliferative response in an acute-phase serum sample collected from a patient with STSS, indicat-

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ing that the sample contained an unknown SAg (28). Mitogenic activity was also detected in serum samples from mice infected with a SAg-producing *S. pyogenes* strain (29).

Since these reports, several novel streptococcal SAgs have been identified, including streptococcal mitogenic exotoxin Z (SMEZ; 30), SMEZ-2, SPE-G, SPE-H (31), SPE-J, and SPE-I (32), all possessing typical SAg features and are highly mitogenic on human T cells. In addition, several variants of SMEZ showed significant antigenic variation (33). These findings suggest that, in addition to SPE-A and SPE-C, one or more of these novel toxins might be involved in STSS.

All known streptococcal SAgs (with the exception of SMEZ, SPE-G, and SPE-J) are localized on mobile DNA elements (34). As a consequence, each GAS isolate usually carries the genes for SMEZ, SPE-G, and SPE-J, plus a certain combination of other *sag* genes. Not much is known about the control of *sag* gene expression, but a recent study indicates that an unknown host factor is involved in the control of SPE-C expression (35)

We analyzed serum samples from 19 patients with severe streptococcal infections for mitogenic activity to identify bioactive SAgs and find a correlation between SAg activity and disease severity. In addition, we genotyped the matching streptococcal isolates from these patients for all known streptococcal *sag* genes and tested them for their ability to produce SAg protein in vitro.

Material and Methods

Patient Serum Samples and Streptococcal Isolates

We included serum samples from all 21 patients referred to the Hammersmith Hospital's Infectious Diseases service from November 1994 to November 2000 who had microbiologically confirmed invasive GAS disease and required hospital admission. Two patients who used intravenous drugs were subsequently excluded to reduce the risk for bloodborne viruses. Aliquots of serum (residual to serum required for clinical purposes) were separated from blood drawn for clinical purposes and frozen immediately at –70°C before testing for mitogens or antibodies. Samples were obtained at the point of admission to hospital (at initiation of antibiotic therapy) and then on sequential days during treatment up to a maximum of 10 days. Streptococcal isolates were cultured directly from blood or tissue, identified by the hospital diagnostic laboratory, and then cultured once in Todd Hewitt broth before immediate freezing in 15% glycerol and before growth for SAg analysis. All 19 patients had invasive streptococcal disease; patients with STSS were identified by using standard criteria (1–3) (Table). The study was approved by the Hammersmith Hospital Research Ethics Committee.

Toxin Proliferation Assay

Human peripheral blood lymphocytes (PBLs) were purified from blood of a healthy donor by using Histopaque Ficoll (Sigma Chemical Co., St. Louis, MO) fractionation. PBLs were incubated in 96-well, round-bottom microtiter plates at 10^5 cells per well with RPMI-10 (RPMI with 10% fetal calf serum [FCS]) containing varying dilutions of recombinant toxins. After 3 days, 0.1 μCi [^{3}H] thymidine was added to each well and cells were incubated for another 24 h. Cells were harvested and counted on a scintillation counter.

Jurkat cells (a human T-cell line) and LG-2 cells (a human B-lymphoblastoid cell line, homozygous for HLA-DR1) were harvested in log phase and resuspended in RPMI-10. One hundred microliters of the cell suspension, containing $1x10^5$ Jurkat cells and $2x10^4$ LG-2 cells was mixed with 100 μL of *S. pyogenes* culture supernatant (undiluted, 1:10, 1:100) on 96-well plates. After incubating overnight at 37°C, 100-μL aliquots were transferred onto a fresh plate and 100 μL ($1x10^4$) of SeI cells (IL-2 dependent murine T-cell line) per well was added. After incubating for 24 h, 0.1 μCi [^{3}H] thymidine was added to each well, and cells were incubated for another 24 h. Cells were harvested and counted on a scintillation counter. As a control, a dilution series of IL-2 was incubated with SeI cells.

PBLs were obtained and stimulated as described under toxin proliferation assay above, with the exception that the 10% FCS was replaced by 5% FCS plus 5% patient serum. All recombinant toxins were used at subsaturating concentrations, which were 0.05 ng/mL (SMEZ-2), 0.1 ng/mL (all other SMEZ variants, SPE-C, SPE-I, SPE-J, streptococcal superantigen [SSA]), 1 ng/mL (SPE-G), 2 ng/mL (SPE-A), and 10 ng/mL (SPE-H).

PBLs from a single donor were used for all tests. We determined the neutralizing response by comparing the T-cell proliferation with a control test using 10% FCS instead of 5% patient serum plus 5% FCS. The relative inhibition was calculated as 1-[cpm (patient serum) per cpm (FCS)]x100.

S. pyogenes isolates were grown overnight in 10 mL of brain heart infusion (BHI) medium (Difco Laboratories, Detroit, MI) at 37°C in 15-mL Falcon tubes without agitation. The cells were spun down and washed, and the genomic DNA was extracted as described previously (33). The purified DNA was resuspended in 50 μL of Tris-EDTA buffer and used for polymerase chain reaction (PCR) with specific primers for the *speA, speC, speG, speH, speI, speJ, ssa*, and *smez* genes as described previously (31–33). In addition, a primer pair specific to a DNA region encoding the 23S rRNA (33) was used as a positive control.

Recombinant forms of SPE-A, SPE-C, SPE-G, SPE-H, SPE-I, SPE-J, SSA, SMEZ-1, and SMEZ-2 were produced in *Escherichia coli* by using the pGEX-2T expression sys-

Table. Summary of the results from *sag* genotyping and SAg in vitro expression

			sag gene/SAg protein production															
Serum	Isolate	Focus	*spe-a*	SPE-A	*spe-c*	SPE-C	*spe-g*	SPE-G	*spe-h*	SPE-H	*spe-i*	SPE-I	*spe-j*	SPE-J	*smez*	SMEZ	*ssa*	SSA	SMEZ[c]
Focal infection/bacteremia with STSS																			
94/31	H292	Fasciitis	-	-	-	-	+	-	+	-	+	++	+	-	+	-	-	-	+
95/02	H293	Fasciitis	-	-	-	-	+	-	+	-	-	-	+	-	+	-	-	-	++
96/2	H297	Peritonitis	+	-	-	-	+	++	-	-	-	-	+	+	+	-	-	-	+
98/5	H325	Fasciitis	+	+++	-	-	+	-	-	-	-	-	+	-	+	-	+	-	+
98/8	H327	Cellulitis	-	-	-	-	+	+	-	-	-	-	+	-	+	_	+	+	-
98/11	H330	Endometritis	+	+++	-	-	+	-	-	-	-	-	+	+	+	-	+	-	+
99/1	H360	Occult bact.	-	-	+	+	+	+	-	-	-	-	+	-	+	-	-	-	++
99/18	H366	Pneumonia	+	+++	-	-	+	-	-	-	-	-	+	++	+	-	-	-	+
20/07	H378	Pneumonia	-	-	-	-	+	-	+	-	-	-	+	-	+	-	+	-	+
Focal infection/bacteremia, no STSS																			
95/8	H295	Cellulitis	-	-	-	-	+	-	-	-	-	-	+	+++	+	-	-	-	++
98/1	H319	Cellulitis	-	-	-	-	+	++	-	-	-	-	+	++	+	-	-	-	++
20/01	H369	Pelvic clot	-	-	-	-	+	-	-	-	-	-	+	+	+	-	-	-	+++
20/04	H370	Thromobosis	-	-	-	-	+	+	-	-	-	-	+	-	+	-	-	-	-
Focal infection, no bacteremia, no STSS																			
97/15	H307	Bursitis	-	-	+	+++	+	-	-	-	-	-	+	+	+	-	-	-	+
97/18	H308	Cellulitis	+	-	-	-	+	-	+	-	-	-	+	-	+	-	+	-	-
97/21	H311	Cellulitis	-	-	-	-	+	-	-	-	-	-	+	-	+	-	-	-	+
97/23	H314	Cellulitis	+	-	-	-	+	+	+	-	+	-	+	+	+	-	-	-	++
97/24	H315	Cellulitis	+	-	-	-	+	-	-	-	-	-	+	+	+	-	-	-	-
97/26	H316	Amnionitis	-	-	+	+++	+	+	-	-	-	-	+	++	+	-	+	-	-

[a]SAg, superantigen; SPE, streptococcal pyrogenic exotoxin; SME, streptococcal mitogenic exotoxin; STSS, streptococcal toxic shock syndrome.
[b]*S. pyogenes* isolates from patients with and without STSS were genotyped by polymerase chain reaction with *sag* specific primers. Concentrated supernatant from the in vitro cultured isolates were analyzed for secreted SAgs by using Western blot analysis with recombinant SAg standards. SMEZ expression was also analyzed by using the more sensitive Jurkat cell assay, which has a threshold of approximately 10 pg/mL. SAg expression by Western blot: -, no detectable protein; +, <2 ng/mL; ++, 2–10 ng/mL; +++, >10 ng/mL. SMEZ expression by Jurkat assay: -, no detectable SMEZ; +, >10 pg/ml (10,000–20,000 cpm); ++, 20,000–30,000 cpm; +++, >30,000 cpm.
[c]Jurkat assay.

tem as described previously (31,33). New Zealand white rabbits were immunized with 50 µg of recombinant protein in 1-mL phosphate-buffered saline and 1-mL incomplete Freund's adjuvants (Invitrogene, San Diego, CA) followed by a booster injection 4 weeks later. The rabbits were bled 10 days after the booster injection. SMEZ-1 and SMEZ-2 were injected as a 1:1 mixture to ensure generation of antibodies against a large panel of the SMEZ variants.

Western Blot Analysis

S. pyogenes isolates were grown overnight in modified BHI medium at 37°C. The medium was prepared by dialyzing 100-mL of 10X concentrated BHI medium against 1 L water. Three grams of glucose, 4 g of Na_2HPO_4, and 10 mL of the dialysate were then added to the solution outside the tubing, which was used to grow the bacteria. Bacterial cells were spun down, and the supernatant was transferred into a new tube and spun at high speed (10,000 rpm) in a Beckman JA20 rotor for 20 min to remove remaining cells. The proteins were then precipitated from the clear supernatant after the addition of 0.15 V of 50% tri-chloracetic acid and centrifugation at 10,000 rpm for 20 min. The precipitated proteins were resuspended in PBL to 1/50 of the original volume and mixed with an equal volume of 2x sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The pH was readjusted by blowing ammonia vapor onto the sample until the color changed from yellow back to blue. After boiling for 2 min, 10 µL was loaded onto a 12% SDS-PAGE and run along a protein standard (50 ng, 25 ng, 10 ng, and 2 ng of the appropriate recombinant SAg).

The proteins were blotted onto a Hybond-c extra nitrocellulose membrane (Amersham Life Sciences, Little Chalfont, UK) by using Western transfer buffer (10% methanol, 150 mM glycine, 25 mM tris-HCl pH 8.5) and a Semi-phor semi-dry blotter (Hoefer Scientific Instruments, San Francisco, CA). The membranes were blocked with 5% milk powder (Anchor, Auckland, New Zealand) in TBST (120 mM NaCl, 10 mM Tris-HCl ph8, 0.05% Tween 20) for 30 min at room temperature, before incubation with the appropriate rabbit anti-SAg antiserum (1:5000 in TBST) overnight at 4°C. The blots were developed by using the ECL Western Blotting Analysis System (Amersham Biosciences, Little Chalfont, UK).

Results

Mitogenic Activity in Samples from Patients with STSS

Nineteen acute-phase serum samples from patients with severe invasive streptococcal disease with STSS (n=9) and without STSS (n=10) were analyzed for mitogenic activity. Serum samples 96/2 and 99/1 both induced a high proliferative response when tested in a PBL-stimulation assay, which suggests that they might contain a SAg. Both patients had bacteremia and STSS. Peritonitis was diagnosed in patient 96/2, who recovered after 2 weeks in the intensive-care unit; TSS was diagnosed in patient 99/1, who died on the day of admission.

To identify the SAg responsible for the mitogenic activity, PBLs were stimulated with serum 96/2 and serum 99/1, respectively, together with rabbit antibodies against recom-

binant forms of SPE-A, SPE-C, SPE-G, SPE-J, or SMEZ (Figure 1A). Addition of anti-SMEZ antibodies resulted in 59% and 68% inhibition of the mitogenic activity of serum samples 96/2 and 99/1, respectively. Antibodies against SPE-J inhibited PBL stimulation of serum 96/2 by 51% but had no substantial effect on the serum 99/1 induced activity. Anti–SPE-A, anti–SPE-C, and anti–SPE-G antibodies did not substantially inhibit the activity in both serum samples, except for a slight inhibition (18%) of serum 96/2 activity by anti–SPE-G antibodies.

The antisera were selective against their individual target SAg. Anti–SPE-J and anti-SMEZ antiserum were added to PBLs stimulated with various recombinant SAgs (SPE-A, SPE-C, SPE-G, SPE-H, SPE-I, SPE-J, SMEZ-1, SMEZ-2, and SSA), and results showed that anti–SPE-J antibodies exclusively inhibited the rSPE-J activity, whereas anti-SMEZ antibodies inhibited the activity of rSMEZ-1 and rSMEZ-2 (Figure 1B). To quantify the levels of SAg in 96/2 and 99/1 serum samples, a comparison was made against a standard PBL proliferation response for recombinant SMEZ-1 and rSPE-J. Five percent of each serum resulted in 33,000–34,000 cpm after ^{3}[H]-thymidine uptake, which is equivalent to 1–10 pg/mL of rSMEZ-1 or rSPE-J (Figure 2). The 99/1 activity was titratable and still detectable at 0.05% serum. Insufficient 96/2 serum prevented a similar dilution assay.

STSS Patient 96/2 and Seroconversion to SMEZ

Sequential serum samples from STSS patient 96/2 up to day 9 after admission to hospital were analyzed for clearance of mitogenic activity. Figure 3 shows that the highest mitogenic activity occurred 1.5 days postadmission (serum 96/2-2). At day 2 postadmission, an 80% reduction of activity had occurred, and by day 3 after admission SAg activity was undetectable. Patient 99/1 died on the day of admission, which prevented sequential serum analysis.

The sequential serum samples from patient 96/2 allowed us to analyze for the development of neutralizing anti-SAg antibodies by using patient serum to inhibit the activity of recombinant SAgs in a PBL-stimulation assay (Figure 4). On day 3, patient 96/2 had neutralizing antibodies against SPE-A, SPE-C, SPE-I, and SSA but undetectable levels of protective antibodies against SMEZ-1, SMEZ-2, SPE-G, and SPE-H. By day 9 after admission (sample 96/2-10), the serum contained high titers of neutralizing antibodies against SPE-A, SPE-C, SPE-I, SSA, SMEZ-1, and SMEZ-2, and a moderate anti–SPE-H titer. No antibodies against SPE-G were detected. These results show seroconversion for SMEZ-1 and SMEZ-2 antibodies in the STSS patient 96/2, adding further evidence that SMEZ was the predominant SAg causing STSS in this patient.

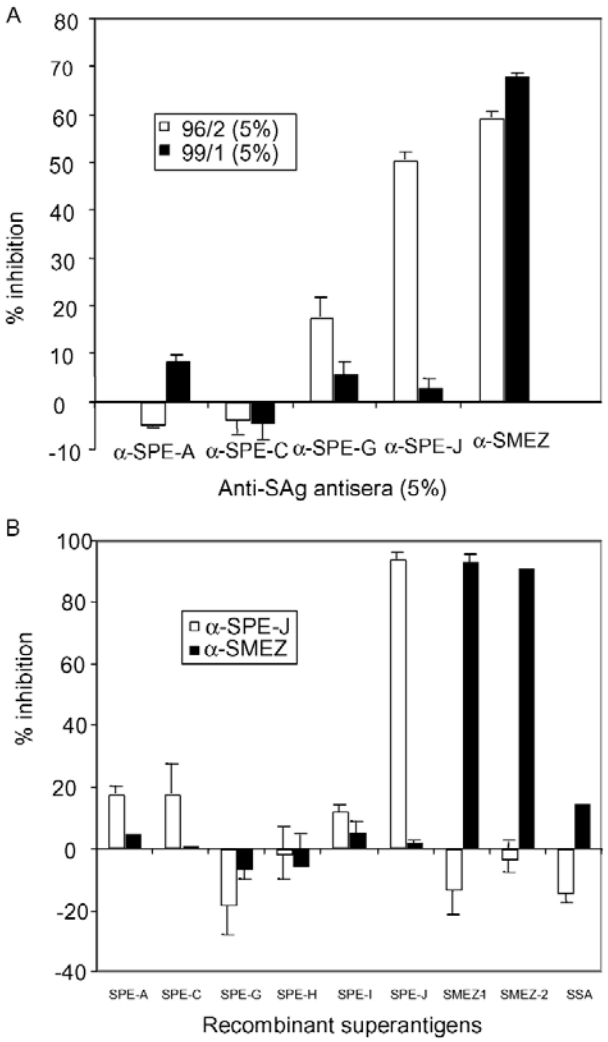

Figure 1. Inhibition of mitogenic activity in sera 96/2 and 99/1 with anti-superantigen (SAg) antisera. A) Peripheral blood lymphocytes (PBLs) were stimulated with 5% patient serum in the presence of 5% anti-SAg antiserum or 5% fetal calf serum (FCS) only. After 3 days, 3[H]-thymidine was added, and PBLs were incubated for another 24 h, before being washed and counted. The results were blotted as percentage of inhibition with specific anti-SAg serum compared to FCS. Antistreptococcal pyrogenic exotoxin (SPE)-J antiserum inhibited the mitogenic activity of serum 96/2 by 51%, while antistreptococcal mitogenic exotoxin (SME) Z antiserum inhibited the activity in 96/2 by 59% and the serum in 99/1 by 68%. B) The specificities of the anti–SPE-J and anti–SMEZ sera were demonstrated by stimulating PBLs with recombinant SAgs in the presence of 5% antiserum. SSA, streptococcal superantigen.

Addition of serum 96/2-4 or 96/2-10 selectively increased the mitogenicity of SPE-J by twofold compared to the FCS control. This selective increase suggests that the serum might contain a substance that selectively synergizes SPE-J activity, which prevented any evaluation of the anti–SPE-J antibody titer in the two sequential serum samples.

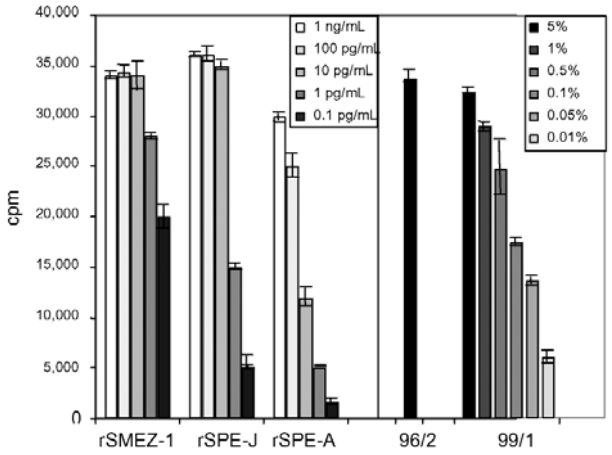

Figure 2. Mitogenic activity of acute-phase serum samples 96/2 and 99/1 compared to recombinant superantigens (SAgs). Peripheral blood lymphocytes were stimulated for 4 d with various dilutions of recombinant SAg or acute-phase serum sample 99/1. No dilution was carried out for 96/2 because of limited amount of serum. Five percent of each of the patient serum samples showed a proliferative response equal to 1–10 pg/mL of recombinant streptococcal pyrogenic exotoxin J or recombinant streptococcal mitogenic exotoxin 1. Serum 99/1 still showed significant mitogenic activity at 0.05%. SME, streptococcal mitogenic exotoxin; SPE, streptococcal pyrogenic exotoxin.

Serum 96/2-10 (9 days after admission) was analyzed for protective antibodies against SMEZ-1, -2, -3, -4, -5, -7, -8, -9, -13, -16, -20, -21, and -22 and found to neutralize the activity of all tested SMEZ variants (data not shown). Inhibition of approximately 95% was seen with all SMEZ variants, except SMEZ-2 (88%), SMEZ-16 (78%), and SMEZ-22 (85%), indicating that challenge with a single SMEZ variant resulted in a cross-reactive antibody response against all SMEZ variants.

Matching GAS isolates from all 19 patients were genotyped for *sag* genes. The frequencies were 100% (*smez, speG, speJ*), 36.8% (*speA*), 31.6% (*ssa*), 26.3% (*speH*), 15.8% (*spC*), and 10.5% (*speI*) (Table). We observed no difference in *sag* gene frequencies between patients with STTS and patients without STSS. The *smez* alleles of both GAS strains isolated from patients 96/2 and 99/1 were analyzed by DNA sequencing and identified as *smez-1* (H297) and *smez-16* (H360).

All GAS isolates were grown in liquid culture, and the supernatants were analyzed for secreted SAgs by Western blot using rabbit antisera against individual recombinant SAgs (Table). The selectivity of each antiserum was tested with the complete panel of recombinant SAgs in Western blots, and no cross-reactivity was observed (data not shown). SPE-A was expressed in substantial amounts only from isolates from patients with STSS (isolates H325, H330, and H366). Isolates from patients without STSS that carry the *speA* gene (H308, H314, and H315) had undetectable levels of SPE-A. However, patients infected with the SPE-A–producing strains did not show any mitogenic activity in their acute-phase serum samples. In contrast, GAS isolated from the patients 96/2 and 99/1 (H297 and H360) produced only small amounts of SMEZ in vitro despite the relatively large amounts detected in the acute-phase serum samples. In vitro–produced SMEZ could not be detected in Western blots, indicating a concentration of <1 ng/mL, and could only be detected using the more sensitive Jurkat cell proliferation assay that has a lower sensitivity threshold (approximately 10 pg/mL).

Discussion

Over the last 2 decades, a large increase in GAS-mediated severe invasive disease has occurred (5,24,25). Streptococcal SAgs have been implicated in STSS and other severe streptococcal infections. Evidence for SAg involvement in these diseases derived from studies showing higher frequencies of *speA* and *speC* genes in severe disease isolates compared to nonsevere disease isolates (2,22–25), and reports of strong proliferative responses in the acute-phase serum samples from a patient with STSS (28) and from mice infected with a SAg-producing *S. pyogenes* strain (29).

In this study, we analyzed 19 acute-phase serum samples from patients with severe streptococcal disease with STSS (n=9) and without STSS (n=10) for mitogenic activ-

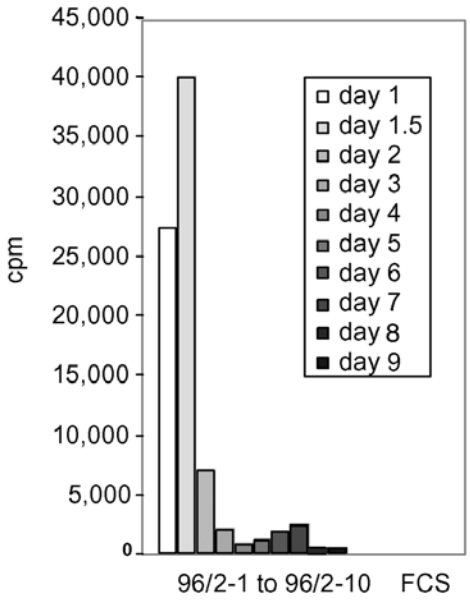

Figure 3. Clearance of the mitogenic activity in sequential sera from patient 96/2. Peripheral blood lymphocytes were stimulated with 5% of acute-phase and sequential serum samples from patient 96/2. The mitogenic activity reached the highest point on day 1.5 after admission to hospital and dropped sharply on day 2. No substantial activity was found in sequential serum samples from day 3 on (samples 96/2–4 to 96/2–10). FCS, fetal calf serum.

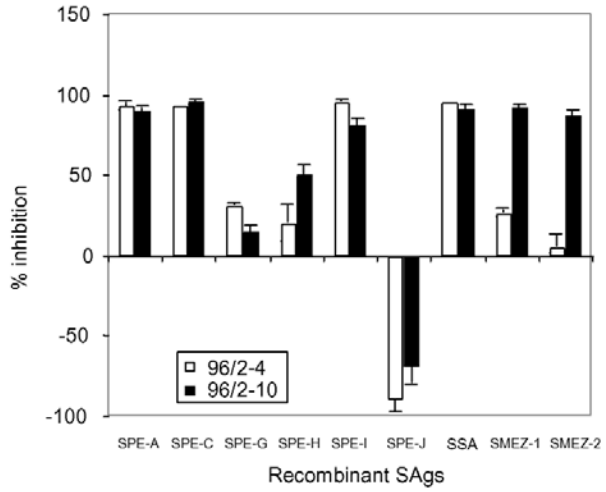

Figure 4. Seroconversion of patient 96/2 against streptococcal superantigens (SAgs). Peripheral blood lymphocytes were stimulated with various recombinant streptococcal SAgs in the presence of serum 96/2–4, 96/2–10, or fetal calf serum only. The columns show the percentage of inhibition of recombinant SAgs by neutralizing antibodies in patient serum samples. The sequential serum on day 3 showed a complete lack of neutralizing antistreptococcal mitogenic exotoxin (SME) Z antibodies, while serum 96/2–10 converted to a high anti-SMEZ antibody titer. Both sera enhanced the mitogenic activity of recombinant streptococcal pyrogenic exotoxin J, which suggests the presence of an unknown synergistic factor. SPE, streptococcal pyrogenic exotoxin; SSA, streptococcal superantigen.

ity and detected SMEZ in samples from 2 STSS patients (96/2 and 99/1). One of the serum samples (96/2) also contained detectable amounts of SPE-J.

The mitogenic activity in the two serum samples was equivalent to 1–10 pg/mL and 10 pg/mL of SMEZ-1 or SPE-J, which is sufficient to trigger maximal T-cell activation in PBL-stimulation assays. However, this concentration remains an estimate as inhibitory effects of serum components were not defined.

The sequential serum of the surviving patient (96/2) showed no protective anti-SMEZ antibodies on day 3 after admission (serum 96/2–4) but substantial levels at day 9 (serum 96/2–10), which suggests a direct role of this toxin in the STSS of this patient. Challenge with SMEZ-1 (the SMEZ variant produced by GAS isolate H297 from patient 96/2) resulted in a broad neutralization response against the complete range of SMEZ variants. We have shown previously that some variation in healthy blood donors exists in neutralizing antibodies to SMEZ variants because of antigenic variation (33). Whether broad-spectrum anti-SMEZ responses are a result of a few cross-reacting antibodies that were raised against a single SMEZ variant has yet to be established, as does whether it results from infection by multiple GAS strains carrying different *smez* alleles over a certain period. Our results indicate the production of cross-reactive SMEZ antibodies in patient 96/2. However, this response might not reflect a general response, and SMEZ concentration in the blood might play a critical role.

Analysis of the 19 matching GAS isolates showed no bias in *sag* genotype between STSS isolates and non-STSS isolates. Furthermore, the results are consistent with the *sag* gene frequencies previously observed in 39 *S. pyogenes* isolates from New Zealand patients with sore throat (n=29), wound infection (n=4), acute glomerulonephritis (n=1), otitis (n=1), endometritis (n=2), rheumatic fever (n=1), and skin ulcer (n=1) (36). The only substantial difference was the frequency of the *speC* gene, found in 42% of New Zealand isolates and 15.8% of London isolates. These results suggest that genotyping of *sag* genes alone is not predictive for type or severity of streptococcal disease.

We found no substantial difference in SAg production in cell cultures between STSS and non-STSS strains, with the exception of SPE-A, which was only expressed in STSS strains and suggests a tight association of SPE-A in some cases of STSS. These data are consistent with data from other groups that showed a link between SPE-A expression and severe streptococcal disease (17,22,23). Evidence points to very different regulation of SAg production between in vitro culture and in vivo infection. SMEZ-1, for example, was undetectable in cultures of isolate H297 except by the sensitive Jurkat proliferation assay, yet the patient with this GAS isolate had high serum levels of SMEZ-1, indicating that SMEZ production is tightly regulated. Kazmi et al. showed the in vivo induction of the *speA* gene by using a micropore Teflon diffusion chamber implanted subcutaneously in BALB/c mice (37). Broudy et al. observed an induction of SPE-C after they co-cultured a *speC*-carrying isolate with a human pharyngeal cell line (35). Some, if not all, SAgs can likely be induced or upregulated by an unknown host factor.

Despite the fact that SMEZ is poorly expressed in vitro while most other SAgs are frequently produced in culture, SMEZ appears to be the predominant bioactive circulating SAg at the time of admission. This observation is in keeping with recent findings using isogenic *smez*+ and *smez*- bacteria in a mouse model (29).

We attempted to correlate streptococcal disease and disease severity with the production of a particular SAg. Although the patient cohort is small, no direct correlation between disease severity and *sag* genotype/SAg production is evident; other factors likely contribute to STSS severity. Importantly, host factors may enhance the production of particular SAgs and, in addition, the immunogenic background of the host may contribute to the severity of SAg-mediated invasive streptococcal disease, as recently reported (38). Although the overall potential role of SAgs in severe streptococcal disease remains elusive,

our results directly implicate SMEZ in the onset of STSS in at least two patients. This presence of a particular bioreactive SAg in the blood of patients with a reemerging potentially fatal disease is new and provides a platform for further investigation of the role of this potent SAg in disease pathophysiology.

This work was supported by the Health Research Council (HRC) New Zealand, the Royal Society of New Zealand (Marsden Fast Start), the University of Auckland (Early Career Research Excellence Award), and the Medical Research Council, UK.

Dr. Proft is a senior research fellow in the Department of Molecular Medicine and Pathology, School of Medicine, University of Auckland, New Zealand. His major interests are bacterial superantigens and their molecular interaction with major histocompatibility complex class II antigen and T-cell receptor.

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Hazards of Healthy Living: Bottled Water and Salad Vegetables as Risk Factors for Campylobacter Infection

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Campylobacter is the most common cause of bacterial gastroenteritis worldwide, yet the etiology of this infection remains only partly explained. In a retrospective cohort study, we compared 213 sporadic campylobacter case-patients with 1,144 patients with negative fecal samples. Information was obtained on food history, animal contact, foreign travel, leisure activities, medical conditions, and medication use. Eating chicken, eating food from a fried chicken outlet, eating salad vegetables, drinking bottled water, and direct contact with cows or calves were all independently associated with infection. The population-attributable fractions for these risk factors explained nearly 70% of sporadic campylobacter infections. Eating chicken is a well-established risk factor, but consuming salad and bottled water are not. The association with salad may be explained by cross-contamination of food within the home, but the possibility that natural mineral water is a risk factor for campylobacter infection could have wide public health implications.

Campylobacter is the most commonly reported bacterial cause of foodborne infection in the Western world and affects more than 2 million people in the United States each year (1). In England and Wales, over 50,000 campylobacter cases are reported each year and show no signs of a decline in incidence (2). For every case reported to laboratory surveillance, another seven cases are estimated to occur in the community, suggesting that from 0.5% to 1.0% of the United Kingdom's population is infected annually (3). Although the infection usually causes a mild, self-limiting illness, serious sequelae, including Guillain-Barré syndrome and death, occur in approximately 1 in 1,000 and 1 in 20,000 infections, respectively (1). Many national food safety agencies, such as the Food Standards Agency in the United Kingdom, have set goals of reducing food poisoning. To achieve these goals, a much clearer understanding of the etiology of campylobacter infection will be necessary.

In spite of the frequency of campylobacter infections, the cause has proved elusive. Recognized outbreaks are rare and are usually caused by contaminated water, milk, or poultry (4,5). However, these food products explain only a small proportion of sporadic cases, and the source of infection is unaccounted for in >60% of U.K. campylobacter cases (6,7). Several case-control studies of risk factors for sporadic campylobacter infection have been performed in the United Kingdom (6–10), but many unanswered questions remain. We conducted a retrospective cohort study that involved mailing a questionnaire to the patient at the time the fecal specimen was received by the laboratory to investigate the cause of sporadic campylobacter infection in the community.

Methods

The study population included all persons living in the Cardiff area who consulted their general practitioner for gastrointestinal symptoms and subsequently submitted a diagnostic fecal sample for microbiologic testing from January 1 through December 31, 2001. Cardiff Public Health Laboratory is the sole laboratory providing a diagnostic microbiology service for the area. All specimens were cultured for *Campylobacter* spp., *Salmonella* spp, *Shigella* spp., and *Escherichia coli* O157 and examined for ova and parasites, by standard methods. Follow-up specimens from the same patient (<4 weeks after the previous specimen submission date); specimens received from hospital wards and other sites were excluded from study. The study was approved by the local research ethics committee.

Immediately upon receipt of the specimen at the laboratory (next working day), a questionnaire, together with an explanatory letter and a postage-paid envelope, was mailed to the patient. Patients who had not responded within 1 week were sent a reminder letter and provided with another questionnaire on request. The questionnaire asked about basic personal details, including age, sex,

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employment status, occupation, details of illness, and details of household contacts. It included sections on foreign travel, food and drink eaten, animal contact (pets and farm animals), outdoor leisure activities (gardening, walking, visits to parks or farms, fishing, swimming, and sports), and questions on specific medical conditions and medication (antacids, H2 antagonists, and antibiotics). The food history covered meat and fish, poultry and eggs, vegetables (raw vegetables, leaf vegetables [e.g., lettuce], salad vegetables [e.g., tomato], and prepared salads [e.g., coleslaw]), fruit, milk and dairy products, drinking water (tap water, bottled water, and other sources), and eating out (type of restaurant or takeaway). Participants were asked to respond yes or no and, to the question of exposure for tap water, to indicate the number of glasses drunk per day. All questions related to exposure in the 7 days before the onset of symptoms, except for those on antibiotics, which concerned the month before illness onset.

Case-patients were defined as any patient, not associated with an outbreak, who submitted a fecal sample that was positive for *Campylobacter* spp. on microbiologic culture. Case-patients were compared with patients whose samples were negative on culture and microscopic examination. Patients with an alternative microbiologic diagnosis were excluded (unless they had dual infection with campylobacter).

Initial univariate analysis was performed with Epi Info software (v. 6.04; Centers for Disease Control and Prevention) to calculate maximum likelihood estimates for Mantel-Haenzel odds ratios (OR) with exact 95% mid-p confidence limits. Continuous variables were analyzed using the t test or Mann-Whitney U test, as appropriate. All reported p values are two sided. Multiple logistic regression models were constructed with Stata software (v. 6, Stata Corporation, College Station, TX). Risk factors were selected a priori on biologic grounds and grouped into four exposure categories: food and drink consumption; animal contact; leisure activities, including foreign travel; and medical history. Logistic regression models were first constructed for risk factors within each exposure category (adjustment A). We then fitted a model that combined all the independent risk factors (for which the Wald test p value for the adjusted OR was <0.10) from the four exposure categories (adjustment B). Finally, to detect any residual confounding, we fitted all personal factors with a p value of <0.10 (age group, presence of a child <5 years of age in the household, and employment status). Of these, only age group interacted significantly with the other terms as tested by goodness of fit and was therefore included in the final model (adjustment C). The population-attributable fraction for each risk factor associated with campylobacter infection was calculated by using methods described by Greenland and Drescher (11).

Results

Questionnaires were sent to 2,694 eligible patients; fecal samples from 346 (12.8%) were positive for *Campylobacter* spp. (including 4 dual infections: 3 with salmonella infection, 1 with giardiasis). No campylobacter outbreak occurred during the study period. Ninety-one patients (3.4%) were positive for other organisms (42 *Salmonella* spp., 20 *Giardia lamblia*, 12 *Cryptosporidium* sp., 7 *Clostridium difficile*, 2 *Shigella* sp., 2 *E. coli* O157, 1 amoebic dysentery, and 5 other parasites); these were excluded from further analysis. Responses were received from 213 (61.6%) of 346 persons with campylobacter infection and 1,144 (50.7%) of 2,257 persons with negative specimen results. Median delay in response (from date questionnaire sent to date questionnaire returned) was 6 days (range 2–73 days) for case-patients and 7 days (range 1–77 days) for non-case-patients (Kruskal-Wallis H 1.81, p=0.18).

Personal Factors and Symptoms

Case-patients (median 43 years of age, range 0–80 years of age) were significantly older than non-case-patients (median 36 years of age, range 0–100 years of age) (Kruskal-Wallis H 5.31, p=0.02) (Table 1). Non-case-patients were also more likely to come from a household that included a child <5 years of age (even after adjusting for the age of the respondent), although not more likely to report prior diarrheal illness in a household contact. Case-patients were more likely to report symptoms than non-case-patients, particularly fever (OR 3.19; 95% confidence interval [CI] 2.36 to 4.31), muscle aches (OR 3.13; 95% CI 2.32 to 4.22), and abdominal pain (OR 3.40; 95% CI 2.32 to 5.12. Nearly all case-patients and most non-case-patients had diarrhea, but case-patients (18.3%) were more likely than non-case-patients (11.8%) to report blood in the stool (OR 1.67; 95% CI 1.12 to 2.46).

Food and Drink Consumed

Case-patients were more likely to report eating meat, including beef, pork, and ham; poultry products, including chicken and eggs; and a variety of uncooked vegetables and fruit, including lettuce, other salad vegetables (cucumber, tomatoes, etc.), preprepared salad (coleslaw, etc.), and fresh or frozen berries. An association existed with drinking bottled water (OR 1.98; 95% CI 1.48 to 2.67) and between infection and drinking cold tap water (OR 1.51; 95% CI 1.06 to 2.18) but not with drinking cold milk. Neither tap water nor milk consumption showed a dose response relationship. Case-patients were more likely to have eaten out in the 7 days before illness onset, particularly at a fried chicken outlet, sandwich bar, or other unspecified restaurant.

Table 1. Comparison of personal and household factors in campylobacter case-patients and non-case-patients

	Case-patients (n=213)		Non-case-patients (n=1,144)			
Variable	No.	(%)	No.	(%)	OR (95% CI)[a]	p value
Female	99	(46.5)	504	(44.1)	1.10 (0.82 to 1.48)	0.56
Age group						
0–14 y	26	(12.2)	328	(28.7)	Reference	
15–44 y	84	(39.4)	323	(28.2)	3.28 (2.06 to 5.23)	<0.001
45–64 y	72	(33.8)	231	(20.2)	3.93 (2.44 to 6.35)	<0.001
≥65 y	30	(14.1)	255	(22.3)	1.48 (0.86 to 2.57)	0.16
Employment status						
Employed	92	(43.2)	352	(30.8)	Reference	
Full-time student	27	(12.7)	82	(7.2)	1.26 (0.77 to 2.06)	0.36
Caring for home and family	15	(7.0)	84	(7.3)	0.68 (0.38 to 1.23)	0.21
Other	45	(21.1)	453	(39.6)	0.38 (0.26 to 0.56)	<0.001
Unemployed	10	(4.7)	31	(2.7)	1.23 (0.58 to 2.61)	0.58
Long-term illness	16	(7.5)	84	(7.3)	0.53 (0.24 to 1.14)	0.29
Mean no. of other people in household (median, range)	3.0	(3) (1-12)	3.2	(3) (1-36)		0.98[b]
Child <5 y of age in the household	28	(13.1)	326	(28.5)	0.38 (0.25 to 0.57)	<0.0001
Mean no. of children <5 y of age in household (median, range)	0.21	(0) (0-5)	0.41	(0) (0-5)		<0.0001[b]
Other ill person in the household	15	(7.0)	112	(9.8)	0.70 (0.39 to 1.20)	0.26
Mean no. of other ill people in household (median, range)	0.09	(0) (0-3)	0.15	(0) (0-6)		0.20[b]
Child caregiver	3	(1.4)	25	(2.2)	0.64 (0.15 to 1.94)	0.61[c]
Food handler	8	(3.8)	61	(5.3)	0.69 (0.31 to 1.41)	0.43

[a]OR, odds ratio; CI, confidence interval.
[b]Mann-Whitney U test.
[c]Fisher exact test.

Animal Contact, Leisure Activities, and Medical History

Case-patients were no more likely than non-case-patients to report pet ownership or contact with other people's pets. Non-case-patients were more likely to own a pet rabbit, though this association was weaker after adjusting for age. Case-patients were more likely to have gone walking, to have visited a farm, or to report contact with cows or calves in the 7 days before illness, though the number of persons exposed to cows was very small. No difference existed in history of recent foreign travel. In respect to medical history, case-patients were no more likely than non-case-patients to suffer from diabetes or indigestion, or to be taking antacid or ulcer medicines but were less likely to report preexisting bowel disease or to have taken antibiotics in the month before onset of illness.

Multivariate Analysis

After adjustment for other variables within each of the four exposure groups (adjustment A), several independent risk factors were identified (Table 2). After combining all these variables (adjustment B), eating frozen fish, eggs, or berries; having milk delivered to the home; eating out at a Chinese restaurant or takeaway; and walking were dropped from the model as they made no independent contribution to the outcome. In the final model (adjustment C), five variables were identified as independent risk factors for campylobacter infection: eating chicken, eating salad vegetables other than lettuce (e.g., tomatoes, cucumber), drinking bottled water, eating out at a fried chicken outlet, and contact with cows or calves (Table 3). Eating lamb, owning a pet rabbit, a history of lower bowel problems, and having had antibiotics in the month before illness all showed a protective effect. The combined population-attributable fraction for the five independent risk variables associated with campylobacter infection was nearly 70%. The highest attributable fractions were for eating chicken (31%), eating salad (21%), or drinking bottled water (12%).

Discussion

Our study identified five risk factors for campylobacter infection that, if taken together, could account for most sporadic cases. Most important was eating chicken in the 7 days before onset of illness. Two other risk factors, not previously described, could also potentially account for a sizeable proportion of case-patients: eating salad vegetables such as tomatoes or cucumber and drinking bottled water.

The study used a retrospective cohort design that included all patients submitting fecal specimens through their general practitioner to a single laboratory. This design controls for patient characteristics associated with physician-consulting behavior and may also minimize recall bias associated with using healthy controls. Use of a laboratory study population does, however, have several disad-

Table 2. Frequency of food exposure, animal contact, leisure activities and medical history in campylobacter infected case-patients and non-case-patients

Exposure	Case-patients (%) (n=213)	Non-case-patients (%) (n=1,144)	Crude OR[a] (95% CI)	Adjusted OR (95% CI)	p value
Food and drink					
Lamb	47 (22.1)	282 (24.7)	0.87 (0.61 to 1.22)	0.67(0.45 to 0.99)	0.046
Frozen fish	53 (24.9)	341 (29.8)	0.78 (0.55 to 1.09)	0.64 (0.45 to 0.93)	0.020
Chicken	177 (83.1)	777 (67.9)	2.32 (1.60 to 3.43)	1.61 (1.03 to 2.50)	0.036
Eggs	141 (66.2)	606 (53.0)	1.74 (1.28 to 2.37)	1.35 (0.95 to 1.92)	0.096
Salad vegetables	159 (74.6)	635 (55.5)	2.36 (1.70 to 3.30)	1.73 (1.09 to 2.74)	0.019
Fresh or frozen berries	51 (23.9)	173 (15.1)	1.77 (1.23 to 2.51)	1.43 (0.95 to 2.13)	0.086
Milk delivered to the doorstep	29 (13.6)	215 (18.8)	0.68 (0.44 to 1.03)	0.60 (0.38 to 0.94)	0.026
Bottled water	114 (53.5)	420 (36.7)	1.98 (1.48 to 2.67)	1.39 (0.98 to 1.96)	0.062
Ate at a fried chicken outlet	22 (10.3)	51 (4.5)	2.47 (1.44 to 4.13)	1.82 (1.00 to 3.30)	0.050
Ate at a Chinese restaurant	23 (10.8)	114 (10.0)	1.09 (0.67 to 1.74)	0.58 (0.33 to 0.99)	0.048
Animal contact					
Own a pet rabbit	7 (3.3)	89 (7.8)	0.40 (0.17 to 84)	0.46 (0.20 to 1.05)	0.066
Had contact with cows or calves	5 (2.3)	6 (0.5)	4.55 (1.27 to 15.74)	5.44 (1.05 to 28.1)	0.043
Leisure activities					
Walking (>15 min)	162 (76.0)	712 (62.2)	1.93 (1.38 to 2.72)	1.92 (1.34 to 2.73)	<0.001
Medical history					
History of lower bowel problems	21 (9.9)	197 (17.2)	0.53 (0.32 to 0.83)	0.55 (0.34 to 0.90)	0.018
Antibiotic in month before illness	11 (5.2)	160 (14.0)	0.34 (0.17 to 0.61)	0.34 (0.18 to 0.65)	0.001

[a]Adjusted for potential confounders within each exposure group; OR, odds ratio; CI, confidence interval.

vantages. Non-case-patients probably represent a group whose illnesses have disparate cause. Many may have had viral gastroenteritis since this is known to be common in the community and is not detectable by routine laboratory testing. This fact would explain why symptoms reported by non-case-patients were milder. Non-case-patients were also significantly more likely than case-patients to report a history of lower bowel problems, suggesting that some had pre-existing disease that might predispose to non-infectious diarrhea. Antibiotic use in the month before onset of illness was also more common in non-case-patients, and symptoms in these persons may therefore be a side effect of antibiotic treatment. Persons with pre-existing bowel problems may have atypical dietary habits, but neither a history of bowel problems nor of antibiotic use should affect the accuracy of food histories. The multivariate analysis controlled for both these variables.

The most consistent finding in studies of campylobacter infection etiology has been an association with eating chicken. This finding has been described in three previous U.K. studies (6,7,9), and in studies from the United States (12–16), Scandinavia (17–19), the Netherlands (20), Switzerland (21), and New Zealand (22,23). However, the relationship with chicken is sometimes only seen for eating undercooked chicken (12,22,23) or eating chicken away from home (8,15,22,23). Recent microbiologic studies of raw poultry in the United Kingdom indicate continuing high levels of campylobacter contamination in chicken and the occurrence of identical subtypes in both chicken and human isolates (24). Our finding of an association between illness and eating chicken or eating from a fried chicken outlet highlights the fact that chicken remains a major risk factor for campylobacter in the United Kingdom and that measures are needed both in the food industry and at home to promote thorough cooking of chicken and to reduce the risk for cross-contamination.

Neither eating salad vegetables nor drinking bottled water has previously been recognized as a risk factor. In our study, both these associations made a significant contribution to the final logistic regression model and could explain a substantial number of campylobacter infections. Both are also biologically plausible. Salad vegetables could be contaminated with campylobacter either before or after the point of sale. Contamination at the source could occur through contaminated soil or contaminated water during harvesting. Salad vegetables are often imported from abroad, and changes in the sourcing of such items could introduce new vehicles of campylobacter infection into the U.K. food chain. For example, contaminated imported lettuce has been identified as a vehicle of infection in recent salmonella and shigella outbreaks in the United Kingdom (25). However, recent extensive sampling of organic fruit and vegetables and ready-to-eat prepackaged salads in the United Kingdom found no pathogens such as *Campylobacter*, *Salmonella,* or *E. coli* O157, suggesting that contamination of such items is either rare or intermittent (26). Two reports on campylobacter outbreaks associated with salad have been published. Both of these involved contamination in the kitchen. The first was a 3-month long outbreak from

Table 3. Multiple logistic regression analysis showing independently associated variables in campylobacter case-patients and non-case-patients[a]

Variable	Adjustment A[b]		Adjustment B[c]		Adjustment C[d]		Attributable fraction (%) (95% CI)
	OR (95% CI)	p value	Odds ratio (95% CI)	p value	OR (95% CI)	p value	
Lamb	0.67 (0.46 to 0.99)	0.05	0.69 (0.48 to 1.00)	0.05	0.68 (0.47 to 0.98)	0.04	
Chicken	1.61 (1.03 to 2.50)	0.04	2.01 (1.35 to 3.00)	0.001	1.79 (1.19 to 2.69)	0.005	31 (9 to 48)
Salad vegetables	1.73 (1.09 to 2.73)	0.02	1.99 (1.40 to 2.82)	<0.001	1.53 (1.06 to 2.21)	0.02	21 (2 to 36)
Bottled water	1.39 (0.98 to 1.96)	0.06	1.57 (1.15 to 2.14)	0.005	1.41 (1.02 to 1.95)	0.04	12 (0 to 23)
Ate at a fried chicken outlet	1.82 (1.00 to 3.30)	0.05	2.08 (1.20 to 3.62)	0.01	1.94 (1.10 to 3.42)	0.02	4 (0 to 7)
Had contact with cows or calves	5.44 (1.05 to 28.10)	0.04	3.98 (1.08 to 14.65)	0.04	5.07 (1.30 to 19.74)	0.02	1 (0 to 3)
Own a pet rabbit	0.46 (0.20 to 1.05)	0.07	0.36 (0.16 to 0.81)	0.01	0.37 (0.16 to 0.83)	0.015	
History of lower bowel problems	0.55 (0.34 to 0.90)	0.02	0.48 (0.29 to 0.79)	0.004	0.45 (0.27 to 0.73)	0.001	
Had antibiotic in month before illness	0.34 (0.18 to 0.65)	0.001	0.41 (0.21 to 0.78)	0.006	0.40 (0.21 to 0.77)	0.006	

[a]OR; odds ratio; CI, confidence interval.
[b]Adjustment A, adjusted for potential confounders within each exposure group.
[c]Adjustment B, adjusted for other significant variables from all four exposure groups.
[d]Adjustment C, adjusted for age group.

cucumber served at a salad bar; the outbreak resolved after changes were made in food preparation and storage procedures (27). The second involved salad prepared by a food-handler who exhibited symptoms of campylobacter infection (28). In a recent review of outbreaks in England and Wales (including five from campylobacter) linked with salad vegetables and fruit, cross-contamination was also the most frequently identified contributory factor (25). The association we observed was specific to items such as tomatoes and cucumber that require extensive handling during preparation and often the use of a chopping board, rather than with lettuce or with salads bought preprepared. This finding suggests that salad most likely gets cross-contaminated during food preparation.

Natural mineral water is usually obtained from springs and occasionally from borehole sources. In Europe, legislation requires that mineral water be free from parasites and pathogenic organisms but, unlike tap water, it may not be treated in any way that might alter its chemical composition (29). A variety of organisms, including coliforms, can be found in mineral water and will survive for a considerable length of time, particularly in uncarbonated water supplied in plastic bottles or bottled by hand (30). To our knowledge, campylobacter has not been identified in mineral water, but this may simply be because testing for campylobacter is rarely undertaken. Mineral water has, however, been identified in the past as a vehicle of transmission during a cholera epidemic (31) and as a potential source of typhoid fever in travelers (32). More recently, a study of diarrhea in HIV-infected persons found symptoms were significantly associated with drinking bottled or filtered water, whereas drinking tap water was protective (33). Drinking bottled water has also recently been identified as a possible risk factor for campylobacter infection acquired abroad (34), and for *Campylobacter coli* infection (35). These findings suggest that bottled water could, given the right circumstances, provide a vehicle of transmission for campylobacter.

A small proportion of cases were explained by contact of personswith cows or calves. This occurred exclusively within the context of a farm visit and probably reflects the urban context of our study. This association, though apparently uncommon, is entirely plausible. Occupational contact with animal feces (8), living on a farm (16,19), and contact with cattle (16,23) have all been previously described as risk factors for campylobacter infection. Healthy beef and dairy cattle both excrete campylobacter (36,37), and molecular evidence suggests a link between campylobacter in the farm environment with those causing disease in the community (38).

Our study confirms that eating chicken still plays an important role in the cause of campylobacter infection. It also identifies two potentially important new risk factors that merit further investigation: salad vegetables (and the associated risks of cross-contamination in the home) and bottled natural mineral water. Cross-contamination in the domestic kitchen is potentially preventable, but we need to know how it happens and what interventions are most effective at reducing the risk. Bottled water is a $35 billion worldwide industry (39). In the United States, 1.7 billion gallons of natural mineral water were consumed in 2000 (39). Consumption is also increasing dramatically in the United Kingdom (by approximately 10% each year), and approximately 300 million gallons of bottled water are now consumed annually (40). Consequently, increased illness from contamination of bottled water could be considerable. More studies of the microbiologic quality of natural mineral waters are required, and these should include testing for *Campylobacter* spp.

Acknowledgments

We thank clerical staff at the Public Health Laboratory Service Communicable Disease Surveillance Centre (Wales) and the Cardiff Public Health Laboratory for their assistance with the study, and all the patients who participated.

This work was funded by a grant from the Public Health Laboratory Service Small Scientific Initiative Fund, grant no. 2001042.

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Escherichia coli O157 Exposure in Wyoming and Seattle: Serologic Evidence of Rural Risk

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We tested the hypothesis that rural populations have increased exposure to *Escherichia coli* O157:H7. We measured circulating antibodies against the O157 lipopolysaccharide in rural Wyoming residents and in blood donors from Casper, Wyoming, and Seattle, Washington, by enzyme immunoassay (EIA). EIA readings were compared by analysis of variance and the least squares difference multiple comparison procedure. Rural Wyoming residents had higher antibody levels to O157 LPS than did Casper donors, who, in turn, had higher levels than did Seattle donors (respective least squares means: 0.356, 0.328, and 0.310; $p<0.05$, Seattle vs. Casper, $p<0.001$, rural Wyoming vs. either city). Lower age was significantly correlated with EIA scores; gender; and, in rural Wyoming, history of bloody diarrhea, town, duration of residence, and use of nontreated water at home were not significantly correlated. These data suggest that rural populations are more exposed to *E. coli* O157:H7 than urban populations.

Escherichia coli O157:H7 is an important human pathogen. This organism can affect humans in a variety of ways, ranging from asymptomatic carriage (1) to diarrhea, bloody diarrhea (the most common manifestation of illness in culture-proven cases), and the postdiarrheal thrombotic microangiopathy, hemolytic uremic syndrome (HUS) (2). Infections with *E. coli* O157:H7 in the Pacific Northwest of the United States have been endemic (3) and epidemic (4). Vehicles transmitting this pathogen include unpasteurized milk and juice (5,6), undercooked beef (7), drinking water (8), and contact with infected persons (9).

Data from the Centers for Disease Control and Prevention (CDC) demonstrate higher incidences of *E. coli* O157:H7 infections in rural counties in the United States than in urban (Paul Mead, unpub. data). Worldwide, rural populations have been postulated to be at greater risk for exposure to *E. coli* O157:H7 by virtue of increased exposure to animals or their excreta in Scotland (10,11); dairy farm visits have been implicated as a source for infection in Finland (12) and the United States (13); and animal contacts are a risk factor for the development of HUS in Switzerland (14). Serologic studies from Canada demonstrated higher frequencies of antibodies to the O157 lipopolysaccharide (LPS) side chain among residents of rural areas compared to residents of urban areas (15), and in Wisconsin children, manure and sheep contact were recently demonstrated to be risk factors for O157 seropositivity (16). Taken together, these data suggest more intense or more frequent human exposure to *E. coli* O157:H7 in nonurban areas.

Populations in the Pacific Northwest and Rocky Mountain states provide an opportunity to assess the frequency of exposure to *E. coli* O157:H7 through serologic studies. Antibodies to the O157 LPS follow natural infection with *E. coli* O157:H7 (17) and are believed to be quite specific (18) because they are rarely found in healthy people. Thus, circulating antibodies to the O157 LPS are potential markers of population exposure to *E. coli* O157:H7. We therefore attempted to assess the distribution of antibodies to this antigen in three different populations, encompassing a gradient of population density.

Methods

Study Participants

Participants were selected for inclusion in this study if they were >16 years of age, weighed >54 kg, and participated in voluntary cholesterol screening in several rural western Wyoming towns (population A), or donated blood to the Wyoming State (population B) or Puget Sound (population C) blood banks, and provided informed consent. The Institutional Review Boards of the Children's Hospital

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and Regional Medical Center (Seattle, Washington) and the University of Wyoming (Laramie, Wyoming) approved this study before participants were enrolled.

Population A consisted of 485 residents of Star Valley, Wyoming. This valley has extensive agricultural land usage and consists of a series of small towns along U.S. Highway 89 in Lincoln County in the northwestern part of the state; town populations range from 100 to 1,200 residents. One of these towns had an *E. coli* O157:H7 outbreak in 1998 (19). During a local health fair conducted in the towns of Afton, Thayne, and Alpine during May 1999, participants donated 5 mL of blood during phlebotomy for screening to detect hyperlipidemia and answered a questionnaire regarding age, gender, treated versus nontreated domestic water supply, history of bloody diarrhea, location of sampling, and town of residence.

Population B was composed of 196 blood donors at United Blood Services (UBS), Casper, Natrona County, Wyoming. UBS, Wyoming's only blood bank, obtains most of its blood from donors residing within Casper, the second largest municipality in the state (population 49,644 residents [20] in 52.8 km^2 [Mike Jun, pers. comm.]). This site was chosen for study because of its presumed intermediate intensity of exposure to agriculture. Volunteers provided 5 mL of whole blood in a separate tube as part of the donation. Gender and age data were available for 127 (65%) of the participants.

Population C consisted of 104 blood donors at the Puget Sound Blood Center, Seattle, Washington, all of whom resided in urban or suburban Puget Sound municipalities (including Seattle and surrounding communities) and provided an additional 5 mL of blood for research. This group was presumed to have less agricultural exposure than populations A or B. Age and gender were recorded for all but two donors. Serum specimens from populations B and C were collected during the spring and summer of 1999.

Cattle and human density data for Lincoln, Natrona, and King Counties are provided in Table 1. These data demonstrate the rural-to-urban human population density and cattle-to-human ratios for the populations chosen. Blood samples were centrifuged within 3 hours of donation; serum samples were separated from the packed erythrocytes and stored at –70°C until assayed.

Detection of Antibodies to *E. coli* O157 LPS

E. coli O157:H7 LPS was purified from strain 86-24 (22) by using phenol extraction (23). Purified LPS underwent electrophoresis in a 12% polyacrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and then was transferred to a membrane and probed with antibodies to the LPS antigen (24). Serum specimens were screened for antibodies to the O157 LPS with an enzyme immunoassay (EIA) (25), modified by CDC. Briefly, in preliminary experiments, the optimal LPS concentrations for coating plates and diluting serum samples were determined by block titration with phosphate-buffered saline (PBS) (0.01 M, pH 7.2) and a known positive human sample. Optimal dilutions of 1:20 for serum and 1:160 of the antigen stock solution (corresponding to 200 µL of a resuspended pellet from a 50-mL overnight culture in Luria broth [26]) were demonstrated (data not shown).

Next, individual wells of an Immulon II plate (Dynex Technology, Franklin, MA) were coated with diluted antigen (100 µL) and incubated (4°C, overnight) to enable the target antigen to adhere to the plates. Then, 150 µL of PBS containing 1% fetal bovine serum and 0.5% nonfat dry milk (PBS-M-FBS) was placed in each well. The plates were then incubated in a blocking step (room temperature, 2 hours) to prevent nonspecific antibody adherence to the plates. Fluid was then removed, and the plates were washed four times with 0.01 M PBS containing 0.05% Tween 20 (PBS-T).

Ten microliters of diluted human serum sample was added to 190 µL of PBS-M-FBS containing 0.05% Tween 20 (PBS-T-M-FBS), placed into wells of the microtiter plates, and incubated (37°C, 1 h). The fluid was then removed, and the plates were washed four times with PBS-T. One hundred microliters of alkaline-phosphatase–labeled goat antihuman immunoglobulin (Ig) G/IgM/IgA (heavy and light chain) (Kierkegaard & Perry Laboratories, Inc., Gaithersburg, MD), diluted 1:10,000 in PBS-T-M-FBS, was then placed in each well and incubated (37°C, 1 h). The plates were then washed four times with PBS-T.

A substrate solution containing 97 mL diethanolamine, 0.2 g sodium azide, 100 mg magnesium chloride-6H_2O, and 800 mL distilled water was adjusted to a pH of 9.8 with 1 M hydrochloric acid. One p-nitrophenyl phosphate

Table 1. Cattle and human density data for Lincoln and Natrona Counties, Wyoming, and King County, Washington (sites of donation)

Characteristics of regions	Population A (Lincoln County)[b]	Population B (Natrona County)[b]	Population C (King County)[b]
Residents	14,793	66,798	1,741,785
Cattle	49,736	61,280	32,806
Cattle/km^2	4.7	4.4	6.0
Cattle/resident	3.4	0.92	0.019
Residents/km^2	1.4	4.8	316.3

[a]Source: reference 20.
[b]Site of collection.

tablet (Sigma Chemical Co., St. Louis, MO) was then added to 5 mL of substrate solution. One hundred microliters of this solution was placed in each well and incubated (room temperature, 25 min). The reaction was halted by adding 50 µL of 3 M sodium hydroxide to each well, and the optical densities of each well were read in a dual wavelength micro-EIA reader at $\lambda = 405$ nm with background correction at $\lambda = 540$ nm (Elx 800, Bio-Tek Instruments, Winooski, VT). Each serum sample was assayed in duplicate, and the values were averaged.

Serum from a patient with HUS caused by *E. coli* O157:H7 and serum from a study participant without known *E. coli* O157:H7 infection in population A were included as duplicates on each plate as positive and negative controls, respectively, and values were averaged. Each plate also contained controls without antigen or primary or secondary antibody. All plates were normalized linearly in relation to the positive control in the first group of serum samples tested.

Analysis

The complete dataset was first studied by analysis of variance (ANOVA, Proc GLM, SAS Institute, Inc., Cary, NC) in a model with EIA readings as the dependent variable, gender and town/city as class-independent variables, and age as a continuous independent variable. Initially, all interactions were included in the model, but interactions not contributing significantly to the model were dropped from subsequent analyses. Multiple comparisons were analyzed by using the protected Fisher least squares differences (LSD) test after confirming that the p value of the model as a whole was <0.05. The data were approximately normally distributed, as demonstrated by a Wilk-Shapiro statistic >0.98 (either for the dataset as a whole or for each region separately, Proc UNIVARIATE, SAS Institute, Inc.) and by visualization of the residuals plot. However, as assumptions of normal distribution of the data are difficult to confirm robustly, the data were also analyzed after transformation of these values into binary form with arbitrarily chosen cutpoints at the 80th and 90th percentiles of the EIA scores or with the entire range of EIA scores categorized at 0.05 increments, using stepwise logistic regression (Proc LOGISTIC, SAS Institute, Inc.) with the same independent variables as described above for the ANOVA (27). Statistically, p values <0.05 were considered significant for comparisons, and p values <0.05 were set as the criterion for entry and for retention into logistic regression models.

Results

We tested 785 serum samples for antibody reactivity. The summary statistics for the O157 EIA are portrayed in the Figure (panel A), and the demographic characteristics

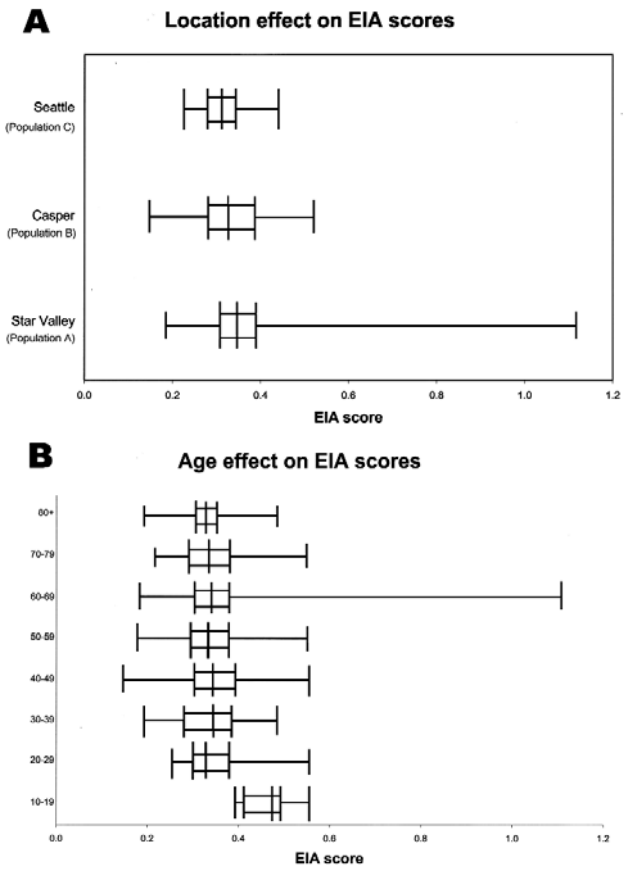

Figure. Box plot analysis for enzyme immunoassay (EIA) values, by populations. X axis represents EIA scores for study participants.Vertical line in each box represents the median for each population. The left and right borders of each box are the 25th and 75th percentiles of each population, respectively. The extensions beyond each box represent the lowest and highest values for each population. Panel A demonstrates the results for each population; panel B demonstrates the results by age.

of each population contributing these serum specimens are provided in Table 1. The average age of the study participants was significantly younger in populations B and C, than in population A. The populations did not differ significantly with respect to gender.

EIA scores were significantly related to both age ($p<0.01$) and population ($p<0.001$) in ANOVA. The effect of age is illustrated in the Figure (panel B). Age and gender information were not available for 35% of population B, but the distributions of EIA scores did not differ between those observations with and without these data, suggesting that the loss of data did not bias the results. The same two variables, age and population, also were significantly ($p<0.05$) associated with EIA score in logistic regression analysis, irrespective of whether the dependent variable modeled was the 80th percentile (Table 2) or the 90th percentile of the EIA score or increased increment of the EIA score (data not shown).

Table 2. Age, gender, history EIA value distribution in each population study, and median and range of OD for the EIA readings

Characteristics of populations	Population A (N =485)	Population B (N =196)	Population C (N =104)
Age (y)			
Mean (SD)	57 (15)	45 (12)	45 (12)
Male: female (not reported)	225:260	56:71 (69)	56:46 (2)
OD (EIA units)			
Least squares mean	0.356[b]	0.328[c]	0.310
Median (range)	0.348 (0.185–1.115)	0.328 (0.149–0.522)	0.312 (0.222–0.441)
EIA, 80th percentile N+ (%)	107 (22.0)	40 (20.4)	5 (4.8)
EIA, 90th percentile N+ (%)	60 (12.4)	19 (9.7)	2 (1.9)

[a]EIA, enzyme immunoassay; OD, optical density; SD, standard deviation.
[b]p<0.001, population A (Star Valley) vs. either Casper or Seattle.
[c]p< 0.05, population C (Seattle) vs. population B (Casper).

Within the rural Wyoming group, the data allowed analysis of O157 LPS EIA values' association with additional variables, including the duration of residence in the area, occurrence of bloody diarrhea, use of a chlorinated water supply at the residence, and town of residence within Star Valley (Table 3). None of these variables was significantly associated with EIA mean values. Residents living in the area for <2 years tended to have higher average EIA values (0.380) than those who resided there for longer periods (2–5 years, 0.346; >5 years, 0.355).

Discussion

These data suggest that rural residents have greater exposure to an antigen or antigens that produce antibodies to the *E. coli* O157 LPS antigen than do urban residents. However, we cannot state with certainty that the precipitating antigen was actually a pathogenic *E. coli* O157:H7. Because the O157 LPS antigen can be expressed by nonpathogenic *E. coli* (28), *Citrobacter freundii* (29), and *E. hermanii* (30), knowing the nature of the antigen during the immunizing event in the participants studied is not possible. Nonetheless, antibodies to the *E. coli* O157 LPS antigen plausibly represent exposure to pathogenic *E. coli* O157:H7, especially as examples exist of asymptomatic carriage of *E. coli* O157:H7 inducing an antibody response to O157 LPS (1,31). We believe, therefore, that this serologic reactivity likely represents actual exposure to pathogenic *E. coli* O157:H7. Also, our assay did not distinguish the classes of antibodies that were reactive in the EIA, so we cannot make estimates about the timing of the exposure based on class of antibody detected. However, IgA, IgG, and IgM antibodies to the O157 LPS are each ephemeral after natural symptomatic infections (32). Thus, the antibodies that we detected in this study quite likely represent recent exposure to the antigen.

The EIA levels were not proportional to cattle density per land area, a value that was similar in each of the three study sites. Thus, human exposure to *E. coli* O157:H7 cannot be attributed simply to cattle presence within counties. However, in rural counties, a higher proportion of residents might be involved in activities that exposed them to *E. coli* O157:H7, including animal contact. Our survey was not designed to measure such exposures within counties. Indeed, cattle-to-human spatial proximity in Ontario is a risk factor for infection in a novel application of livestock density indicators and disease incidence (33). Alternatively, rural residents might have a higher frequency of exposure to wild animals that carry *E. coli* O157:H7, such as deer (34), their excreta, or water that has been contaminated with their excreta. However, if a link between any animal source of *E. coli* O157:H7 and human exposure to this pathogen is to be further investigated, population distributions within counties, proximities of humans to animals and their excreta, and presence of *E. coli* O157:H7 in the environment will all need to be examined to determine the modes of contact.

Table 3. Age, gender, and enzyme immunoassay (EIA) value distribution in rural Wyoming communities, including the median and range of optical densities (OD) for EIA readings

Characteristics of populations	Afton (N=253)	Alpine (N=84)	Thayne (N=148)
Age (y)			
Mean (SD)	56 (15)	55 (12)	61 (14)
Median (range)	56 (16–92)	56 (20–78)	63 (16–92)
Male: female	120:133	39:45	66:82
OD (EIA units)			
Least squares mean	0.357	0.340	0.359
Median (range)	0.349 (0.192–1.115)	0.343 (0.185–0.550)	0.353 (0.230–0.643)
EIA, 80th percentile N+ (%)	69 (27.3)	11 (13.1)	31 (20.9)
EIA, 90th percentile N+ (%)	38 (15.0)	5 (6.0)	16 (10.8)

Our data differ from results of previous studies in terms of statistical analysis and also because we included a gradient of population density. Specifically, our principal analysis did not require assignment of persons to seropositive or seronegative status categories. While some relation might exist between percentage of persons who are designated as having reactive or nonreactive status on the basis of cutoffs and the comparative distribution of serologic reactivities in populations, we believe that, when examining continuous variables, a test that compares values as continuous measurements (such as analysis of variance) provides more information and is less arbitrary than the assignment of categorical positives and negatives. We did, however, also examine proportions above two cutpoints, and the same trends were noted. For unknown reasons, we identified higher EIA scores in younger study participants, whereas in Ontario, IgG antibodies to the O157 LPS were highest in participants in their fifth decade of life (15). Comparing our age-related EIA scores to those reported recently from Wisconsin, where older children had higher seropositive rates, is not possible because the latter study focused on a child population and did not evaluate older Wisconsin residents (16).

We caution against interpreting our data to mean that rural populations are immune to, and thereby protected from, *E. coli* O157:H7 infections. Many study participants had antibody levels that were probably too low to confer protection, even if one assumes that antibodies to this antigen protect against infection. In this regard, an *E. coli* O157:H7 infection, which almost always induces a brisk and high-titer humoral immune response to the O157 LPS antigen, may not confer a permanently protective response, as evidenced by documentations of recurrent infections (35,36). However, in a recent *E. coli* O157:H7 outbreak in the Star Valley, a higher frequency of antibody levels to the O157 LPS among the resident population was proposed as the reason for a lower attack rate among residents than among visitors (19). We also urge against generalizing the trend observed in this study, which is derived from the analysis of only three populations, to all rural populations, without additional, more widespread, studies because the populations studied might not be representative. Nonetheless, our data are consistent with the hypothesis that rural residence carries with it a greater risk for exposure to *E. coli* O157:H7 than does urban residence.

In summary, we have identified an age-dependent, gender-independent, risk for probable exposure to *E. coli* O157:H7 in persons living in rural communities. This exposure frequency is plausibly environmental, rather than foodborne, in origin because food is distributed widely throughout North America. However, the possibility exists that particular food consumption practices, such as drinking raw milk in rural communities, as has been noted in the United Kingdom (37,38), might have been responsible for this exposure. We also cannot exclude the possibility that the differences observed relate to the nature of the participants studied. That is, donors to blood banks might have different exposures than participants in lipid screenings at health fairs. Future studies should attempt to identify the points of exposure to this antigen, confirm that *E. coli* O157:H7 is, indeed, the source of the inciting antigen, and, if it is, minimize human contact with this pathogen.

Acknowledgments

We thank Jennifer L. Falkenhagen for assistance in manuscript preparation, Paul Mead for sharing unpublished data with us, and William Bibb for advice concerning enzyme immunoassay protocols.

The *Escherichia coli* Gift Fund at the Children's Hospital and Regional Medical Center and NIH grant DK52081 support this project.

Dr. Haack is a resident at the Mayo Clinic, Rochester, Minnesota. This work was performed as a thesis project at the University of Washington School of Medicine.

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Characterization of Waterborne Outbreak–associated *Campylobacter jejuni*, Walkerton, Ontario

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The Walkerton, Canada, waterborne outbreak of 2000 resulted from entry of *Escherichia coli* O157:H7 and *Campylobacter* spp. from neighboring farms into the town water supply. Isolates of *Campylobacter jejuni* and *Campylobacter coli* obtained from outbreak investigations were characterized by phenotypic and genotypic methods, including heat-stable and heat-labile serotyping, phage typing, biotyping, fla–restriction fragment length polymorphism (RFLP) typing, and pulsed-field gel electrophoresis. Two main outbreak strains were identified on the basis of heat-stable serotyping and fla-RFLP typing. These strains produced a limited number of types when tested by other methods. Isolates with types indistinguishable from, or similar to, the outbreak types were found only on one farm near the town of Walkerton, whereas cattle from other farms carried a variety of *Campylobacter* strains with different type characteristics. Results of these analyses confirmed results from epidemiologic studies and the utility of using several different typing and subtyping methods for completely characterizing bacterial populations.

An outbreak of *Campylobacter jejuni* in a farming community in southern Ontario, Canada, in 1985 resulted from contamination of well water caused by spring run-off and heavy rains (1). In May 2000, a second waterborne outbreak of *Escherichia coli* O157:H7 and *Campylobacter* occurred in Bruce County, Ontario. Well water serving the town of Walkerton was contaminated by surface water carrying livestock waste immediately after heavy rains (2,3). A detailed microbiologic and epidemiologic analysis of the most recent outbreak may provide insights that could help make this type of outbreak less frequent.

Most sporadic cases of campylobacteriosis are associated with preparation or consumption of poultry products (4). Outbreaks have been associated with consumption of unpasteurized milk or unchlorinated water (5). An estimated 20% of cases of illness caused by *C. jejuni* are due to vehicles of infection other than food, including water (6). Waterborne outbreaks of *Campylobacter* tend to occur in spring or early fall, an association attributed to seasonality of surface water contamination and infection in cattle herds (5). Contaminated water sources have been implicated in outbreaks involving *E. coli* O157:H7 and *Campylobacter* together in Scotland (7) and in New York State (8,9). The former outbreak resulted from sewage contamination of the water supply of a small village in Fife, Scotland. The latter outbreak was associated with contamination of wells at a state fair (10). Excrement from birds and animals, including cattle, has been shown to contaminate surface water supplies used by humans infected with *Campylobacter* (9).

Campylobacter spp. have been found to cause waterborne outbreaks worldwide; such outbreaks are a particular problem in Scandinavian countries where many people drink untreated water from streams and other sources (11). Untreated surface water has also been implicated in *Campylobacter* outbreaks in New Zealand (12,13), Finland (14), England, Wales (15,16), Australia (17), and the United States (18). In Canada, outbreaks have been rarely detected and have been associated with contamination of surface water (19,20) and consumption of unpasteurized milk (21).

In the United States, disease caused by *C. jejuni* or *C. coli* has been estimated to affect 7 million people annually, causing 110–511 deaths and costing $1.2–$6 billion (22). These organisms are responsible for 17% of all hospitalizations related to foodborne illness in the United States, and although associated with a much lower case-fatality rate than *Salmonella* spp. and *E. coli* O157:H7, they account for 5% of food-related deaths (6). Although

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the incidence of *Campylobacter* infections generally appears to be higher in industrialized than in developing nations, some evidence exists that campylobacteriosis may be important from a social and economic point of view (23).

Epidemiologic and microbiologic analyses were undertaken to better understand the circumstances leading to the Walkerton outbreak. *C. jejuni* was isolated from patients associated with the outbreak, and *C. jejuni* and *C. coli* were isolated from animals and animal manure on farms located near the town wells. This work summarizes the phenotypic and genotypic typing results for isolates associated with the outbreak.

Materials and Methods

Epidemiologic Investigations

Identification of the outbreak, definition of cases, and the results of epidemiologic descriptive and cross-sectional studies have been described (2,3). Isolates from persons who did not meet all requirements for the case definition, but who resided in southwestern Ontario and became ill during the period of the outbreak, were also sent to the National Laboratory for Enteric Pathogens (NLEP), Winnipeg, Manitoba, for further analysis. A detailed description of the epidemiologic investigations is in preparation.

Environmental Specimens

Environmental studies related to the outbreak have been described previously (2,3). Initial investigations identified 13 livestock farms within a 4-km radius of the three wells serving the town of Walkerton. From May 30 to June 13, 2000, a minimum of five manure samples per farm were obtained and tested for human enteric pathogens. Bovine rectal swabs and manure were collected from a subset of these farms in follow-up studies on June 13. All specimens were screened for *Campylobacter* spp., and isolates were forwarded to NLEP for further testing.

Processing of Specimens

Patient stool specimens were collected into Cary-Blair transport medium and sent to the Central Public Health Laboratory, Ministry of Health and Long-Term Care, Toronto, Ontario. Specimens from animal manure were collected aseptically in sterile bags and forwarded to the same laboratory. Stools (approximately 1 g) from both sources were added into liquid enrichment medium (LEM) or directly onto charcoal-selective medium (CSM) and incubated at 42°C in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2) for 24 h and 48 h. Cultures in LEM were subcultured to CSM and incubated as indicated above. Isolates submitted to the NLEP were routinely cultured on Mueller-Hinton agar (Oxoid Ltd., London, England) containing 10% sheep blood and stored frozen at –70°C in glycerol peptone water. Isolates were routinely incubated at either 37°C or 42°C in a microaerobic atmosphere.

Identification of Isolates

Colonies suspected of being *Campylobacter* were Gram stained and tested for oxidase, catalase, and hippurate hydrolysis. Presumptive identification of *C. coli* was achieved by the indoxyl acetate test and by determining susceptibility to nalidixic acid (30-µg disk) and cephalothin (30-µg disk). Biotyping was performed as described by Lior (24). In addition to biotyping, the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) identification scheme described by Marshall et al. (25) was used to confirm species identification. Primers specific to *C. jejuni* (25) and to *C. coli* (26) were used to confirm the identity of any "hippurate-negative" *C. jejuni*. Any isolates that were hippurate-negative in the tube test but positive by PCR for the hippuricase gene and negative by PCR for the aspartokinase gene associated with *C. coli* were confirmed by retesting by both methods.

Strain Subtyping

Heat-labile (HL) serotyping was performed by the method of Lior et al. (27). HS serotyping, in which passive hemagglutination was used to detect heat-stable antigens, was performed by the method of Penner and Hennessy (28). Phage typing of isolates was performed as described by Frost et al. (29). Fla-RFLP typing was performed by the method of Nachamkin et al. (30). Numerical type designations from 1–101 were assigned at the NLEP. PFGE was done according to the method of Ribot et al. (31) with *Sma*I and *Kpn*I. The isolates tested by PFGE were the first human and animal isolates to be sent to the NLEP, and testing continued until type characteristics of outbreak strains were identified and the epidemiologic designations of patients involved in the outbreak were confirmed microbiologically. After this, only biotyping, serotyping, and phage typing were used to characterize outbreak strains. Fla-RFLP typing was implemented some time after the outbreak in an attempt to determine the effectiveness of this method for subtyping outbreak strains. All isolates tested by PFGE, and a random selection of isolates not tested by PFGE, were subject to fla-RFLP analysis.

Results

A detailed description of the epidemiologic and environmental investigations is the subject of a manuscript in preparation (A. Ellis, pers. comm.). A total of 532 human stool specimens were tested for *Campylobacter* spp. Stools from 116 persons were positive for the organism, and 11 of these were also positive for *E. coli* O157:H7. Of these 116

strains, 106 were submitted to NLEP for further analysis, along with 20 strains from southern Ontario not directly linked to the outbreak. *Campylobacter* spp. (49 isolates) obtained from animals or manure on 11 of 13 farms tested were also sent to the NLEP for further analysis (2,3). No *Campylobacter* organisms were isolated from the 57 water samples tested.

All 175 isolates were characterized, first by biotyping and serotyping, then by phage typing (Table 1). A subset of 83 isolates was further characterized by PFGE, while 115 isolates were subsequently tested by fla-RFLP typing. *C. jejuni* or *C. coli* were confirmed by using PCR for the hippuricase and aspartokinase genes, a strategy that also allowed the definitive identification of hippuricase-negative (hipp. neg.) *C. jejuni* strains. Five biotypes (I, II, III, IV, and hipp. neg.) were found among the isolates, with biotype II predominating. HS serotyping detected 14 different serotypes among the larger group of 175 isolates. Three HS serotypes were epidemiologically associated with the outbreak (Tables 1 and 2). Most outbreak-associated strains were HS serotype O:2. Phage typing was useful for further strain discrimination, yielding 22 PTs (25 if phage type variants were included) plus two isolates with atypical lytic patterns and two untypeable strains. PT 33 was most commonly associated with outbreak strains, though other phage types were also outbreak-associated. HL serotyping generated 29 types from the group of 175 strains. PFGE divided the 83 strains tested into more than 30 types when both *Sma*I and *Kpn*I were used (Tables 2 and 3). Though fla-RFLP typing produced 22 different types, only 7 were epidemiologically associated with the outbreak. When combined, the results from all phenotypic and genotypic assays created a large number of distinct types (Tables 2, 3, and 4). HL serotyping allowed typing of 150 (86%) of 175 isolates tested. HS serotyping achieved 97% typeability, while phage typing and molecular typing methods typed 99% and 100% of strains tested, respectively.

The characteristics of outbreak strains were derived by correlating the results of phenotypic and genotypic assays. Only biotypes II and hipp.-neg. *C. jejuni* were strongly associated with the outbreak, although 13 biotype III isolates were also identified. Most (99/106; 92%) of the patient isolates epidemiologically associated with the outbreak expressed HS serotype O:2 (Table 2). The most common phage types among all isolates tested were PT 33 (100/175 isolates), PT 13 (12/175 isolates), and PT 1 (10/175 isolates). These phage types were found in isolates epidemiologically associated with the outbreak as well as those that were not, although 82/106 (77%) of patient isolates associated with the outbreak were PT 33 (Table 2). Of the 57 fla-RFLP type 1 isolates characterized, 50 (88%) were epidemiologically associated with the outbreak. PFGE *Sma*I types CASAI.0001, .0002, .0004, and .0011 clustered on the same branch of a dendrogram constructed with PFGE patterns from isolates obtained at the time of the outbreak (Figure 1) and were closely associated with other type characteristics connected with the outbreak, including HS serotype O:2; fla type 1; PT 33; and HL types 125, 128, and UT. Five other isolates considered epidemiologically unrelated to the outbreak had Penner type O:2, fla-RFLP type 1, PT 33, and biotype II. Four of these isolates were HL serotype 125. One strain had the PFGE outbreak type CASAI.0001, CAKNI.0001, and three of the four other strains had outbreak type CASAI.0002, CAKNI.0002. The final strain had PFGE type CASAI.0002 and CAKNI.0036, a PFGE pattern varying from CAKNI.0002 only by two minor bands at the bottom of the gel. In this context, all strains were considered outbreak type 1 strains. A second fla-RFLP type, 34, was closely associated with 11 isolates from both humans and animals, all of which were epidemiologically associated with the outbreak. All strains with fla-RFLP type 34 were HS serotype O:2, hipp. neg., and PFGE type CASAI.0003, CAKNI.0003, although four different HL serotypes and five different phage types were present (Table 4). In addition, one strain with fla-RFLP type 99 was HS serotype O:2, hipp. neg., and PFGE type CASAI.0003, CAKNI.0003. This combination of types and subtypes was considered outbreak type 2 (Table 2). As shown in Figure 2, although fla-RFLP type 99 is more similar to type 1 than type 34, it still differs from type 1 by three bands. Only seven other isolates representing a few other distinct *C. jejuni* types were also considered to be epidemiologically associated with the outbreak (Table 2).

Table 1. Tests used for analysis of *Campylobacter* isolates from Bruce-Grey County Ontario, Spring 2000[a]

Test	No. strains tested	No. types obtained	No. types outbreak associated
Species	175	2	1
Biotype	175	5	3
HS serotype	175	14	3
Fla-RFLP type	115	22	7
Phage type	175	27	14
HL serotype	175	29	13
PFGE type (*Sma*I)	83	30	6
PFGE type (*Kpn*I)	65	17	4

[a]HS, heat-stable; RFLP, restriction fragment length polymorphism; HL, heat-labile; PFGE, pulsed-field gel electrophoresis.

Farms near the town of Walkerton were considered as possible sources of bacteria causing the outbreak. Sampling of animals on 11 farms yielded a number of *C. jejuni* and *C. coli* isolates (Table 3). Isolates of outbreak type 1 were found from cattle on farm 2 and farm 14, whereas the second outbreak type was found in cattle on farm 2. A variety of different strains were obtained from other farms, although none expressed characteristics of the two major outbreak types.

Table 2. Characteristics of *Campylobacter jejuni* strains from human patients

Species	Biotype[a]	HS type	Fla-RFLP type	PFGE type using *Sma*I	PFGE type using *Kpn*I	No. strains	Outbreak type
Campylobacter jejuni	II	O:2	1	CASAI.0001	CAKNI.0001	13	1
C. jejuni	II	O:2	1	CASAI.0001	CAKNI.0001	1	NER
C. jejuni	II	O:2	1	CASAI.0002	CAKNI.0002	8	1
C. jejuni	II	UT[a]	1	CASAI.0002	CAKNI.0002	1	1
C. jejuni	II	O:2	1	CASAI.0002	CAKNI.0002	2	NER
C. jejuni	II	O:2	1	CASAI.0002	CAKNI.0036	1	NER
C. jejuni	II	O:2	1	CASAI.0002	CAKNI.0003	1	1
C. jejuni	Hipp.neg.[a]	O:2	1	CASAI.0002	CAKNI.0003	1	1
C. jejuni	II	O :2	1	CASAI.0004	CAKN1.0001	2	1
C. jejuni	II	O:2	1	CASAI.0011	CAKNI.0001	1	1
C. jejuni	II	O:2	1	ND[a]	ND	14	1
C. jejuni	II	O:2	ND	ND	ND	20	1
C. jejuni	II	O:2	ND	ND	ND	1	NER
C. jejuni	II	O:2	ND	ND	ND	28	1
C. jejuni	II	UT	ND	ND	ND	1	NER
C. jejuni	Hipp. neg.	O:2	34	CASAI.0003	CAKNI.0003	9	2
C. jejuni	Hipp. neg.	O:2	34	CASAI.0003	CAKNI.0003	1	2
C. jejuni	II	O:1,44	2	CASAI.0012	CAKNI.0012	1	NER
C. jejuni	I	O:3	ND	ND	ND	1	NER
C. jejuni	II	O:4 complex	93	ND	ND	2	Not defined
C. jejuni	II	O:4 complex	93	ND	ND	1	NER
C. jejuni	II	O:4 complex	ND	ND	ND	1	NER
C. jejuni	II	O:4 complex	90	CASAI.0030	CAKNI.0024	1	NER
C. jejuni	I	O:4 complex	94	ND	ND	1	NER
C. jejuni	II	O:4 complex	90	ND	ND	1	Not defined
C. jejuni	II	O:17 complex	1	ND	ND	1	NER
C. jejuni	I	O:11	91	CASAI.0029	CAKNI.0026	1	NER
C. jejuni	III	O:17 complex	5	ND	ND	1	Not defined
C. jejuni	I	O:17 complex	99	ND	ND	1	NER
C. jejuni	III	O:21	5	ND	ND	1	Not defined
C. jejuni	IV	O:21	5	ND	ND	1	NER
C. jejuni	II	O:17 complex	4	ND	ND	1	Not defined
C. jejuni	III	O:17 complex	5	ND	ND	1	Not defined
C. jejuni	III	O:17 complex	5	ND	ND	1	NER
C. coli	I	O:34	36	CASAI.0020	CAKNI.0025	1	NER
C. jejuni	II	O:35	92	ND	ND	1	NER
C. coli	I	O:47	82	CASAI.0010	CAKNI.0004	1	NER

[a]HS, heat-stable; RFLP, restriction fragment length polymorphism; PFGE, pulsed-field gel electrophoresis; NER, not epidemiologically related to the outbreak; UT, untypeable; hipp. neg., lack of hippurate hydrolysis in *C. jejuni* strains; ND, not determined

Two of the strains recovered from animals on farm 7 shared some characteristics with isolates from humans epidemiologically associated with the outbreak. A patient isolate with HS serotype O:4 complex (O:13), biotype II, phage type 6, fla type 91, and HL serotype 7 was similar to an animal isolate with HS serotype O:4 complex (O:13), biotype II, phage type 6, fla type 93, and HL serotype UT. Two isolates from humans associated with the outbreak had HS serotype O:4 complex, biotype II, fla type 93, HL serotype 7, and PT 13 or 71. Isolates from animals on farms 1 and 7 were similar but were considered epidemiologically unrelated to the outbreak (2,3).

Methods Used for Strain Characterization

Many isolates were distinguishable by types obtained with only one or two methods, while all other types remained the same. Some strains varied only in the expression of their O:4 complex (O:4, O:13, O:16, O:43, and O:50 [32]) HS serotypes (data not shown). A single patient isolate with serotype O:2, the hipp. neg. *C. jejuni* biotype, and PFGE type CASAI.0003 differed from a group of nine other patient isolates by expressing fla type 99 rather than type 34 (Table 2, Figure 2); several HL serotypes and phage types were found within this group of isolates (Table 4). Two strains with HS serotype O:17 complex (O:17,23,36), and HL serotype 5 had different fla-RFLP

Table 3. Characteristics of *Campylobacter* strains found from cattle on farms during the outbreak

Farm	Species	Biotype	HS serotype	Fla-RFLP type	PFGE type using *Sma*I	PFGE type using *Kpn*I	No. strains	Outbreak associated
1	*C. jejuni*	II	O:1	33	CASAI.0034	ND[a]	1	No
	C. jejuni	II	UT[a]	33	CASAI.0034	ND	1	No
	C. jejuni	II	O:4 complex	90	CASAI.0026	ND	1	No
	C. jejuni	II	O:4 complex	90	CASAI.0030	CAKNI.0024	1	No
	C. jejuni	II	O:4 complex	ND	ND	ND	1	No
	C. jejuni	I	O:18	ND	ND	ND	1	No
	C. jejuni	I	O:18,37	33	CASAI.0013	ND	1	No
	C. coli	I	O:26,30,34	ND	ND	ND	1	No
	C. coli	I	O:34	ND	ND	ND	1	No
	C. jejuni	II	O:35	2	CASAI.0033	ND	1	No
	C. jejuni	II	O:35	ND	ND	ND	1	No
2	*C. jejuni*	II	O:2	1	CASAI.0001	CAKNI.0001	1	Yes
	C. jejuni	II	O:2	1	CASAI.0004	CAKNI.0001	2	Yes
	C. jejuni	II	O:2	1	CASAI.0004	CAKNI.0005	1	Yes
	C. jejuni	II	O:2	1	ND	ND	3	Yes
	C. jejuni	II	O:2	ND	ND	ND	2	Yes
	C. jejuni	II	O:2	1	ND	ND	2	Yes
	C. jejuni	Hipp. neg.	O:2	34	CASAI.0003	CAKNI.0003	2	Yes
3	*C. jejuni*	III	O:38	74	CASAI.0005	CAKNI.0006	1	No
	C. jejuni	III	O:4 complex	95	CASAI.0006	CAKNI.0007	1	No
	C. jejuni	III	O:13,50,65	95	CASAI.0006	CAKNI.0007	1	No
5	*C. jejuni*	II	O:1	97	CASAI.0007	CAKNI.0008	1	No
6	*C. jejuni*	II	O:4 complex	6	CASAI.0017	ND	1	No
	C. jejuni	II	O:18	33	CASAI.0014	ND	1	No
7	*C. jejuni*	II	O:4 complex	93	CASAI.0016	ND	1	No
	C. jejuni	II	O:4 complex	93	ND	ND	1	No
	C. jejuni	II	O:4 complex	90	ND	ND	1	No
	C. jejuni	II	O:4	93	CASAI.0007	CAKNI.0009	1	No
	C. jejuni	II	O:13,O64	93	CASAI.0015	CAKNI.0028	1	No
8	*C. coli*	I	O:25	73	CASAI.0019	ND	2	No
	C. coli	I	O:34	98	CASAI.0018	ND	1	No
9	*C. coli*	I	O:34	36	CASAI.0020	CAKNI.0025	1	No
	C. jejuni	II	O:11	ND	ND	ND	1	No
	C. jejuni	II	UT	52	UT	ND	1	No
10	*C. jejuni*	I	O:11	91	CASAI.0031	CAKNI.0027	1	No
	C. jejuni	II	O:4 complex	6	CASAI.0022	CAKNI.0029	1	No
	C. jejuni	II	O:35	2	CASAI.0021	ND	1	No
	C. jejuni	II	UT	2	CASAI.0021	ND	1	No
12	*C. jejuni*	II	O:2	101	CASAI.0025	ND	1	No
	C. coli	I	O:34	36	CASAI.0024	ND	1	No
	C. jejuni	I	O:35	2	CASAI.0023	ND	1	No
14	*C. jejuni*	II	O:2	1	ND	ND	1	No

[a]HS, heat-stable; RFLP, restriction fragment length polymorphism; PFGE, pulsed field gel electrophoresis; ND, not determined; UT, untypeable.

types, phage types, and biotypes. Two similar bovine isolates from farm 1 had an identical fla-RFLP type (type 90) and similar HS serotypes (O:4 complex) but had different phage types, HL serotypes, and PFGE types (Table 3). All strains from farm 7 carried some combination of types that included HS serotypes O:4 complex, either fla-RFLP types 90 or 93, HL serotype 7 or UT, and a number of phage types. Most phage types did not show a 1:1 correlation with types obtained with other methods or with the outbreak (Table 4). HL serotyping appeared to be more discriminatory than the other methods used, although HL serotypes did not appear to change at random from types obtained with all other methods. The HL types associated with the outbreak were found only in isolates with HS serotype O:2 (Table 4).

Discussion

Phenotypic and molecular typing methods together support the hypothesis that bacteria entered the Walkerton municipal water supply from neighboring farms and impli-

Table 4. Variability of phage typing (PT) and heat-labile (HL) type in outbreak strains 1 and 2

PT	HL Type	No. isolates: fla-RFLP and PFGE types NT	fla-RFLP type 1, PFGE types NT	fla-RFLP type 1, PFGE strain 1 types	fla-RFLP types 1 & 99, PFGE strain 2 types	Total no. isolates
Outbreak strain type 1[b]						
13	128	-	-	1	-	1
31	110	-	-	1	-	1
33	4	1	1	-	-	2
	[4,125]	1	2	-	-	3
	100	1	1	-	-	2
	110	1	-	-	-	1
	112	1	1	-	-	2
	[112,125]	2	2	-	-	4
	125	20	-	19	-	39
	128	9	2	1	-	12
	[125,128]	-	3	1	-	4
	UT[a]	10	-	3	-	13
33 var.	UT	-	-	1	-	1
35	125	-	-	1	-	1
40	125	1	-	-	-	1
64	128	-	1	-	-	1
UT	128	1	-	-	-	1
Outbreak strain type 2[c]						
13	128	-	-	-	2	2
	UT	-	-	-	2	2
14	UT	-	-	-	1	1
28	4	-	-	-	1	1
	100	-	-	-	1	1
71	4	-	-	-	2	2
	100	-	-	-	1	1
Total		48	13	28	10	99

[a]RFLP, restriction fragment length polymorphism; PFGE, pulsed-field gel electrophoresies; ND, not determined; UT, untypeable.
[b]HS O:2 or UT; fla RFLP type 1 or ND[a]; PFGE types CASAI.0001, .0002, 4, 11, ND, CAKN.0001, 2, 3, ND
[c]HS O:2; fla-RFLP type 34 or 99; PFGE types CASAI.0003, ND, CAKNI.0003, ND

cate farm 2 as the major source of outbreak strains. This conclusion was consistent with hydrogeologic models in which runoff from heavy rains swept *Campylobacter* spp. and *E. coli* O157:H7 bacteria from farm 2 into the vicinity of well 5, where they gained access to the well and were distributed through the town's water supply (2,3). A few isolates indistinguishable from the outbreak strain were recovered from patients not epidemiologically associated with the outbreak, suggesting that these patients might indeed have been associated with the outbreak. The outbreak case definition would not exclude sporadic cases occurring at the same time as the outbreak. These isolates could represent cases of secondary transmission or patients having an indirect association with the outbreak that were not identified during the epidemiologic investigation.

Isolates from some patients who were epidemiologically associated with the outbreak produced molecular subtyping results that differed from the outbreak type, suggesting that these bacteria might have been acquired from a source other than well 5 or that they may have been present on farm 2 adjacent to well 5 but not detected. These organisms could have entered the water supply near well 6, though that well was not as susceptible to contamination as well 5 (3). If well 6 was involved, isolates with the types found on other farms (e.g., farm 7) near the well should have comprised a higher proportion of outbreak strains. Patients could have acquired the organisms through direct or indirect contact with animals or persons from farms or from some other common source. Strains with characteristics similar to these non-O:2 strains were often not found on farms in the Walkerton area. Though the outbreak affected many residents in this area, it may have occurred against a background of sporadic cases.

The diversity seen among the *Campylobacter* isolates is in striking contrast to the single *E. coli* type infecting Walkerton outbreak patients and in cattle on farm 2 (2,3, data not shown). Furthermore, during the New York state fair outbreak, a single *Campylobacter* PFGE type predominated (9). Isolates from a point source outbreak caused by tuna salad had the same HS serotype, HL serotype, and biotype (33). Routine surveillance of *Campylobacter* by HS serotyping and phage typing identified a single type

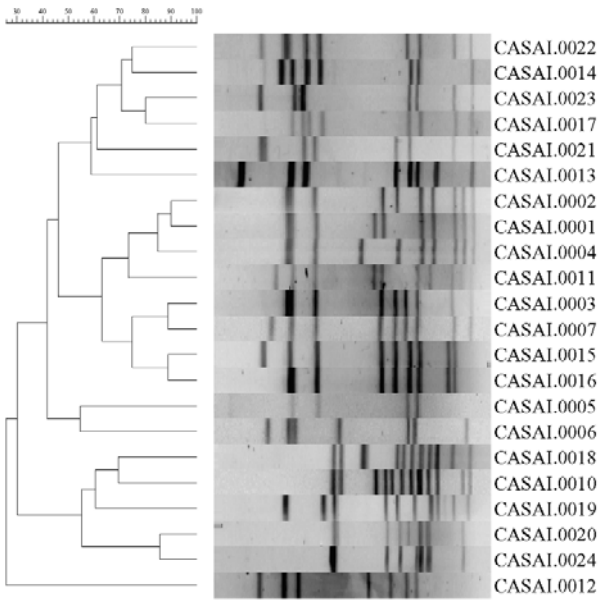

Figure 1. Dendrogram showing pulsed-field gel electrophoresis of *Campylobacter* isolates using *Sma*I.

that caused an outbreak associated with stir-fried food in the United Kingdom (34). HS serotype, ribotype, DNA profile, and PFGE all showed the same profile in isolates obtained from a 6-week continuous source waterborne outbreak in a town in Denmark (35). A damaged sewer line was implicated in this outbreak. In contrast, of 25 outbreaks investigated by Frost et al. (36), isolates with only one PT and HS serotype were found in 13 outbreaks and multiple types (up to eight) in 12 outbreaks. The diversity of HS serotypes and PFGE types encountered in Walkerton may therefore be somewhat unusual, while the diversity of HL serotypes and phage types is consistent with information in the literature. This diversity could be the result of inclusion of strains or types that were not outbreak related or from the heterogeneity of types at nearby farms. Existing data do not allow us to determine which of these hypotheses is correct.

Continuous, comprehensive databases of molecular subtyping data for *Campylobacter* species have not yet been developed in Canada. Whether the Walkerton outbreak types are rare types or common types in Canada is not known. This uncertainty makes interpretation of the data more difficult and highlights the need for continuous surveillance of pathogens to support the interpretation of typing and fingerprinting data.

Different methods performed quite differently for characterizing strains. Fla-RFLP typing and Penner serotyping appeared to group strains into larger clusters, which was useful for identifying outbreak-associated strains. Results from these two methods together would have allowed good predictions about whether a *Campylobacter* isolate should be included in the outbreak investigation. A close correspondence has previously been found for *fla*A-RFLP types and *Sma*I PFGE types which, together with HS serotypes, were found to identify *C. coli* clonal lines having epidemiologic significance (37). HS serotype O:2 appears to be a common strain of *Campylobacter* (38) and is found frequently in isolates from both humans and cattle (39,40). Additional information from fla-RFLP typing may therefore be necessary for more definitive discrimination. Several isolates belonged to the HS O:4 complex, with each antigen expressed variably in individual strains. Strains expressing this complex predominated on farms 3 and 7 (Table 3) and were also found in three isolates from patients (Table 2). Only isolates from farm 7, near well 6, had the fla-RFLP types 90 and 93 in common with patient isolates. The sources of infection of these patients was not clear, although well 6 probably did not become contaminated (3).

PFGE data correlated well with HS serotyping and fla-RFLP data. A group of closely related PFGE patterns was

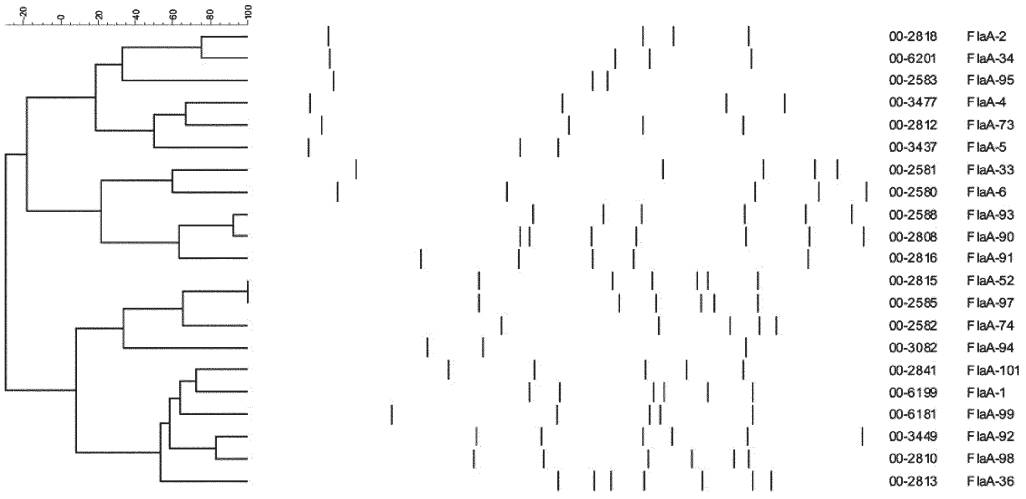

Figure 2. Dendrogram showing *Campylobacter* restriction fragment length polymorphism types.

associated with the outbreak. PFGE was more discriminatory than fla-RFLP typing and HS serotyping, and during the outbreak, additional information had to be collected to associate all five *Sma*I PFGE types with the outbreak. This limited the utility of PFGE for identifying outbreak strains until epidemiologic data were available. Although fla-RFLP typing had a lower apparent discriminatory power compared with PFGE, it was more useful for organizing strains into epidemiologically relevant groups. Close examination of PFGE patterns CASAI.0001, .0002, .0004, and .0011 indicated an underlying similarity, with changes that suggested duplication, insertion, or deletion of DNA from a common ancestral type (Figure 1). Strains with patterns CASAI.0001, .0004, and .0011 all shared a common *Kpn*I restriction pattern (CAKNI.0001). Differences in these patterns are consistent with changes within restriction sites or rearrangements. PFGE pattern CASAI.0002, however, was associated with patterns CAKNI.0002 and CASAI.0003. CAKNI.0002 differs from CAKNI.0001 only in the position of the top band, which is higher in CAKNI.0001 (data not shown). Patterns CASAI.0001, .0004, and .0011 differed from CASAI.0002 by the position of a single larger band in each pattern (Figure 1). These differences are more consistent with the addition of DNA through insertion of exogenous material or duplication of chromosomal loci. Recombination appears to occur frequently within *Campylobacter* species and, with genomic rearrangement, contributes to the genomic instability characteristic of certain strains (39–41). The events causing the PFGE changes seen in these closely related patterns remain to be determined. That such changes may occur at relatively high frequency is suggested by the discriminatory power of PFGE compared with other typing methods.

Phage typing was useful in defining the outbreak strains in early stages of the investigation due to the speed with which results could be obtained. However, the second most common outbreak type isolated, defined by HS serotype O:2, hipp. neg. biotype, fla-RFLP type 34, PFGE CASAI.0003, had several phage types (13, 14, 28, 33 var., 71). Phage types varied independently of the other characteristics measured (Table 4), giving this typing method a higher apparent discriminatory power than HS serotyping, fla typing, or PFGE. Including isolates into the outbreak on the basis of phage type alone,without accompanying epidemiologic data, would have been difficult. This factor may limit the utility of phage typing for detection of outbreaks, though at least one outbreak has been identified on the basis of phage typing and HS serotyping. PT 33 was, however, an effective marker for the most prevalent outbreak type.

HL serotypes 4, [4,125], 100, 112, [112, 125], 125, [125,128], and 128 were almost exclusively associated with outbreak isolates. Though it would have been difficult to identify outbreak strains on the basis of HL serotype alone, this method did help confirm the link between outbreak strains in humans and isolates from farm 2. The 125 and 128 serotypes have been seen infrequently by NLEP and are more "unique" markers than either HS serotype O:2 or phage type 33. Serotype HL 5 was associated with the HS O:17 complex in isolates from human patients and was not found in isolates from any of the farms. The source of these isolates was not determined, though O:17 strains have previously been recovered from poultry (24). HL serotype 7 was associated with the HS O:4 complex discussed earlier, suggesting that the associations between HL and HS serotypes noted previously (23) may not be random. Within the outbreak strain, however, changes in HL serotype appeared to occur more frequently than, and independently from, other type characteristics. HL typing would not have been of use in the identification of the two Walkerton outbreak strains if used in the absence of epidemiologic information. That two HL serotypes could be found, namely types [4, 125], [112, 125], and [125, 128], all of which included HL 125, was interesting. Further characterization of these complex HL serotypes could provide useful laboratory-based epidemiologic information.

In summary, two *Campylobacter jejuni* strains were associated with the Walkerton outbreak through the use of different typing and subtyping methods in combination with epidemiologic data. These methods were useful for defining the scope of the outbreak, for identifying the source of strains, and for tracing the route by which bacteria infected humans. The bacteriologic findings fully support the results of the epidemiologic and hydrogeologic investigations (2,3), which suggest that bacteria from cattle manure were able to enter groundwater after heavy rains and contaminate a well serving the town of Walkerton, subsequently infecting those consuming the water. Some investigators think that adult beef cattle represent a limited threat to water supplies and subsequent transmission of *Campylobacter* to humans (42). However, recent investigations suggest that the environment, as well as cattle and other farm animals, may play an important role in human infection with these organisms (38,39). Studies of the contribution of cattle feedlots and other farm operations to *Campylobacter* contamination of surface waters and watersheds, as well as subsequent human infections, would provide useful information for farm management practices and the protection and management of water resources.

Acknowledgments

We acknowledge the contributions of Shelley Johnson, Jennifer Campbell, and Dave Spreitzer for pulsed-field gel electrophoresis analysis; Walter Demczuk for valuable support for

database management, laboratory surveillance, communication activities during the outbreak, and phage typing; and Louis Bryden for critiquing the manuscript and providing advice.

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Cultural Contexts of Ebola in Northern Uganda

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Technical guidelines for the control of Ebola hemorrhagic fever (EHF) indicate that understanding local views and responses to an outbreak is essential. However, few studies with such information exist. Thus, we used qualitative and quantitative methods to determine how local residents of Gulu, Uganda, viewed and responded to the 2000–2001 outbreak of EHF. Results indicated that Acholi people used at least three explanatory models to explain and respond to the outbreak; indigenous epidemic control measures were often implemented and were consistent with those being promoted by healthcare workers; and some cultural practices amplified the outbreak (e.g., burial practices). However, most persons were willing to modify and work with national and international healthcare workers.

Many emerging disease specialists are sensitive to and acknowledge the potential importance of social science in disease control, but seldom is this perspective considered when organizing response efforts. In part, this situation exists because so little research in this area has been conducted. The special issue on Ebola in The Journal of Infectious Diseases (1) does not include any articles on the behavioral aspects of the disease. However, World Health Organization (WHO) technical guidelines for responding to Ebola hemorrhagic fever (EHF) state that, in conducting epidemiologic surveillance, "Special attention must be given to the actual perception of the outbreak by the community. In particular, specific cultural elements and local beliefs must be taken into account to ensure proper messages, confidence, and close cooperation of the community" (2).

We describe the first systematic sociocultural study of an outbreak of EHF. The outbreak occurred in several locations in northern Uganda in 2000 to 2001. We conducted this research in villages and neighborhoods in and around Gulu during the last month of the outbreak. The field study aimed to: 1) describe local explanatory models of EHF; 2) provide understanding of topics of concern to WHO (i.e., burial practices, patients' fear of going to the hospital, the role of traditional healers in disease transmission); and 3) identify local and international beliefs and practices that enhanced or were detrimental to the control of EHF. An "explanatory model" refers to a person's or culture's explanations and predictions regarding a particular illness. Some of the questions asked when trying to understand an explanatory model include: How do persons refer to the illness? How do they explain it (i.e., cause)? What do they see as appropriate treatments? What do they do to prevent the illness? Patients, physicians, healthcare workers, and local residents in different parts of the world each have explanatory models for different illnesses. Providing care and treatment for a particular disease is often based on negotiating these different models.

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Background

The 2000–2001 Uganda outbreak was one of the largest EHF outbreaks to date, with 425 presumptive cases and 224 deaths (case-fatality rate 53%). Most patients were women (269 or 63%). The earliest reported presumptive case-patient had disease onset on August 30, 2000, and the last case began on January 9, 2001 (3).

The Gulu EHF outbreak was relatively unusual in comparison to other recent EHF outbreaks (e.g., Democratic Republic of Congo [DRC], Gabon) in that the disease affected primarily one ethnic group, the Acholi, and most of the district medical staff and decision makers (e.g., district medical officer, director of health education) were also from this ethnic group. Also, most (60%) EHF cases occurred in the urban area of Gulu town. Gulu District has approximately 470,000 people, primarily Acholi, and 60% of the population live in protected villages because of rebel activity.

Most Acholi are agro-pastoralists and have a social organization strongly influenced by patrilineal descent and patrilocal postmarital residence (4). Other researchers have written about Acholi and other Nilotic peoples' health beliefs (5–7), but none has described cultural responses to epidemic diseases.

Methods

Qualitative and quantitative methods were used. The first 2 weeks of the research emphasized open-ended and

focus group interviews as well as document review (e.g., health education materials, reports). The last few days emphasized the development of systematic questionnaires. Open-ended interviews were conducted with the following: 1) 10 persons and four focus groups in villages or neighborhoods with large numbers of early cases of EHF; 2) 8 persons and one focus group with survivors of EHF (both healthcare workers and community members); 3) four focus groups with male and female elders (two meetings with each gender); 4) 3 persons and two focus groups of children; 5) 4 persons and two focus groups with healthcare workers responsible for the isolation unit and counseling survivors; and 6) 4 persons and one focus group with traditional healers. Focus group meetings usually had 5–8 participants, with the exception of the survivor focus group meeting, which had 35 participants.

Questionnaires were administered to 85 Gulu High School students 15–21 years of age (all members of three senior classes; 33 men, 52 women). Precoded questionnaires were administered to 49 adults in Gulu (25 women, 24 men; an adult from every third house from two randomly selected Gulu neighborhoods) and 60 EHF survivors (22 men, 38 women; all survivors were located through a survivors' organization). We also examined existing documents, such as field reports and health education materials used in the outbreak (e.g., posters, brochures, music cassettes, videos).

Results

Explanatory Models

Table 1 summarizes three primary explanatory models identified by the Acholi. The third model is biomedical. Biomedical models have existed in the area for ≥100 years, and all Acholi know these models well and use them often. In the early phases of the outbreak, many families, thinking the disease was a bacterial infection or malaria, turned to tetracycline or chloroquine. Most early case-patients went to the hospital seeking biomedical treatment. The biomedical model for EHF was introduced in late October by the Ugandan Ministry of Health. The health education program was multidimensional (e.g., posters, radio shows, videos, brochures) and transmitted this model effectively. However, by the time the EHF biomedical model was introduced, local people had already used two other indigenous explanatory models.

Both of these models require an understanding of the concept of *jok*, which is common to many Nilotic-speaking peoples, including the Acholi. *Jok* are spirits or gods (8). Many different types of *jok* exist; they have names and reportedly are often found near bodies of water, mountains, and natural salt licks for cattle. *Jok* are generally benevolent, as they provide and control resources, but they can also cause harm if they are not respected. Deference to and respect for others are central values in Acholi life, and spiritual life reflects and reinforces these values. These spirits are like elders in the community; the Acholi listen to what they have to say, do what they say without question, and give them gifts to show respect.

Traditional healers (*ajwaka*), who are primarily women, obtain their powers to heal from specific *jok* that they have acquired through time. Most healers acquire as many as 10 such spirits during their lifetime. Each spirit has a name and a specific kind of knowledge (e.g., treatments for mental confusion or infertility).

At first, many persons treated the symptoms of EHF as a regular illness and sought a variety of both biomedical (i.e., malarial drugs or antibiotics) and indigenous cures (i.e., herbs, traditional healers). In late September 2000, the heads of families in neighborhoods with many deaths asked a traditional healer to locate poisons (*yat*) in and around the lineage household that might be causing the illness and death (Table 1, first explanatory model). The healers used their *jok* and special spears to locate poisoned objects (e.g., roots, bones) in the neighborhood. The healer's *jok* was also called on to communicate with the spirit associated with a particular poison and to determine if burning the object and sacrificing goats and sheep was necessary to demonstrate respect. Once the poison was removed and respect demonstrated, the healers said the deaths would stop. But the deaths continued. *Yat* removal is not cheap; each EHF-affected family paid 150,000 Ugandan shillings (U.S.$88), four to five goats or sheep, and one chicken (about half the annual income for a rural Acholi family). The families pointed out that in addition to the enormous loss of loved ones, an incredible amount of family assets was lost in trying to treat the ill.

In early October, residents began to realize that this outbreak was more than a regular kind of illness and began to classify it as *two gemo* (two [illness] gemo [epidemic]), the second explanatory model in Table 1. *Gemo* is a bad spirit (type of *jok* that comes suddenly and causes a mysterious illness and death in many people within a very short period of time). *Gemo* reportedly comes like the wind in that it comes rapidly from a particular direction and affects many people, but the wind itself does not necessarily bring it. Acholi have experienced other types of *gemo* (e.g., measles and smallpox). Forty-nine of 50 adults interviewed indicated a belief that Ebola was a type of *gemo*. The term *two gemo* was also used in health education posters and music.

Gemo is said to be mysterious in that it just comes on its own, but several people indicated that it comes because of lack of respect and honor for the gods. Elders indicated that in the past, lack of respect for *jok* of *tura* (hills, mountains, bodies of water) was the major cause of *gemo*.

Table 1. Explanatory models for Ebola hemorrhagic fever (EHF) among the Acholi

Terms	*Yat*	*Gemo*	Disease of contact; Ebola
Description	"Medicine" or substance that enters the body and causes illness	Bad spirit that comes suddenly and rapidly and effects many people	EHF, biomedical description
Signs and symptoms	Starts with pain inflammation but can have many other signs in later stages	Mental confusion, rapid death, high fever	High fever, vomiting, headache
Causes	Bad "medicine" (poison) goes into body	Lack of respect for *jok*, sometimes no reason	Filovirus, but host reservoir unknown
Transmission	Step on it, eat it, catching it, somebody sends, just looking at a person	Physical proximity, easy for *gemo* to catch you	Physical contact with bodily fluids of patients
Pathophysiology	Inflammation and pain in area touched by or location of *yat*	Attacks all of body	Damage to major organs
Treatment	*Tak*—techniques of healers who use their *jok* to identify and remove *yat* from body or environment	Talk to *jok* via traditional healer, give whatever wants, gifts of food to *jok*	None, hydrate (ORS), control vomiting
Prevention and control	Protective bracelets	See protocol in text, *chani labolo, ryemo gemo*	Do not touch patients, barrier nursing
Prognosis	Good if removed from body; otherwise death	Not good, no cure	Not good, no cure
Risk groups	Very smart, successful, salaried people; anybody	Caregivers close to patients (women), families that do not respect *jok*, families that do not follow protocol	Unprotected healthcare workers, caregivers of patients, people that wash or touch dead victims
Political	Infected troops returning from DRC sent to Gulu	Infected troops returning from DRC sent to Gulu	Infected troops returning from DRC sent to Gulu

[a]ORS, oral rehydration salts; DRC, Democratic Republic of Congo.

People talk about *gemo* catching you, so if someone is close to a person with *gemo* it is easier for *gemo* to catch you. Once an illness is identified as *gemo*, a protocol for its prevention and control is implemented that is quite different from the treatment and control of other illnesses.

When an illness has been identified and categorized as a killer epidemic (*gemo*), the family is advised to do the following: 1) Quarantine or isolate the patient in a house at least 100 m from all other houses, with no visitors allowed. 2) A survivor of the epidemic should feed and care for the patient. If no survivors are available, an elderly woman or man should be the caregiver. 3) Houses with ill patients should be identified with two long poles of elephant grass, one on each side of the door. 4) Villages and households with ill patients should place two long poles with a pole across them to notify those approaching. 5) Everyone should limit their movements, that is, stay within their household and not move between villages. 6) No food from outsiders should be eaten. 7) Pregnant women and children should be especially careful to avoid patients. 8) Harmony should be increased within the household, that is, there should be no harsh words or conflicts within the family. 9) Sexual relations are to be avoided. 10) Dancing is not allowed. 11) Rotten or smoked meat may not be eaten, only eat fresh cattle meat. 12) Once the patient no longer has symptoms, he or she should remain in isolation for one full lunar cycle before moving freely in the village. 13) If the person dies, a person who has survived *gemo* or has taken care of several sick persons and not become ill, should bury the persons; the burial should take place at the edge of the village.

From a biomedical perspective, this protocol constitutes a broad-spectrum approach to epidemic control. Isolation and identification of the patient's home and village were emphasized by all groups interviewed, but sexually transmitted and foodborne transmissions were also frequently listed. Elders were adamant that this protocol existed before the arrival of Europeans in the late 1800s. Although historic research is needed to verify this claim, the facts that an indigenous term (*gemo*) is associated with the behaviors, the belief is integrated into the religious system (*jok*), and the protocol is common knowledge to children who do not learn it in school suggest that many rules existed in pre-Colonial times.

Several other ways exist to try to control *gemo*, including driving it away to the Nile by noisemaking (*ryemo gemo*). This procedure was conducted several times during the outbreak and is conducted every December 31 to chase away any potential *gemo* before the New Year begins. Another local custom is *chani labolo,* which consists of wearing a dried banana leaf bracelet for 3 days (for men) or 4 days (for women) to protect and chase away *gemo*. Some healers have *jok* that is supposedly specific for *gemo*, and three of the four traditional healers interviewed indicated their *jok* told them about the impending *gemo* before it arrived (i.e., back in August 2000).

Most local residents saw a political dimension to the explanatory models of EHF (Table 1). Many felt that EHF came from infected Ugandan soldiers returning from DRC. Residents felt that the current government has little interest in the North so when Ugandan soldiers became infected in the DRC, the decision was made to send them to military

bases in Gulu. Although some of the first female victims of EHF had relationships with men in the military, existing epidemiologic evidence does not support the DRC origin hypothesis. The origin of this outbreak is not known. Political dimensions to disease, killer epidemics, in particular, are common. The first author's visit to Gabon during the 1996 EHF outbreak indicated that local people believed that the French military, which had military exercises in the area just before the outbreak, were partially responsible for introducing the disease. In addition, the 2003 Congo outbreak was linked, in part, to activities of Euro-Americans conducting research in a national park there.

Many informants and healthcare workers indicated that fleeing the village or neighborhood was common, in particular in locations with the highest number of infected persons. Fleeing is not an explicit part of the explanatory models but makes some sense because the *gemo* or biomedical models indicate the illness is a rapid killer transmitted by close contact with infected persons.

Most persons involved in this outbreak were familiar with all three explanatory models and did not necessarily see them as contradictory. Healthcare workers emphasized the biomedical model, but many Acholi healthcare workers participated in *ryemo gemo* when it passed through the community. Some persons and villages turned to the *yat* and *gemo* explanatory models but did not hesitate to purchase tetracycline and other medicines to treat cases of EHF. The first two explanatory models may seem strange to international health workers, but they reflect a holistic and social view of illness common to many people in the world. Acholi are aware of the biomedical model but view illness as having social, spiritual, and biological dimensions. The epidemic control protocol is a good example. Family members refrain from sex and quarreling to show respect to *jok* (spiritual) and increase family harmony and peace (social).

Issues of Concern

Funerals and Burials

National and international healthcare workers were concerned that burial practices contributed to the amplification of EHF. A brief study indicated that once a person died, his or her paternal aunt (father's sister) was called to wash and prepare the body for burial. If the father did not have a sister, an older woman in the victim's patriline was asked to prepare the body. Generally, the woman removed the clothes from the body, washed the body, and dressed the deceased in a favorite outfit. At the funeral, all family members ritually washed their hands in a common bowl, and during open casket all were welcome to come up to deceased person and give a final touch on the face or elsewhere (called a love touch). The body was then wrapped in a white cloth or sheet and buried. The person was buried next to or near their household. This practice is the normal system of burial.

However, when disease is classified as *gemo,* burial practices change. The body is not touched and is buried outside or at the edge of the village. The designated caregiver, someone who has survived the outbreak or an older woman, is responsible for washing and preparing the body for burial.

Various activities associated with burial practices contributed to transmission of EHF (Table 2). Washing the body was a possible means of infection for women only, while a touch was a more common means of infection among men. The fact that 63% of the survivors in this study had their first symptoms in October implies that they probably became infected before laboratory tests confirmed EHF and before the disease was designated as a type of *gemo* in many communities. Caregiving, especially by women, contributed substantially to many cases, which explains, in part, why 63% of all presumptive EHF cases in Uganda were in women.

WHO was also concerned that local persons were not coming to the hospital when symptoms first emerged. Healthcare workers theorized that patients were afraid of being buried at the airfield if they died. Persons were running and hiding when the ambulance arrived to take them to hospital. Later interviews indicated, however, that the airfield burial was not the problem. As described in the protocol, once an illness is identified as a killer epidemic, burial at the edge of the village is expected. Rather, sources indicated, many persons ran from the ambulance and did not seek treatment quickly because they feared they would never see their family once they were admitted to the hospital. A fear of Euro-Americans buying and selling bodyparts is common in many parts of central Africa but was especially pronounced in Gulu hospitals because bodies were placed in body bags and taken to the airfield to be buried without relatives being notified. Relatives were not always around at the time of death, and healthcare workers were required to dispose of the body as quickly as possible. The anger and bad feelings about not being informed were directed toward healthcare workers in the isolation unit. This fear could have been averted by allowing family members to see the body in the bag and allowing family members to escort the body to the burial ground.

Table 2. How survivors thought they contracted Ebola hemorrhagic fever (EHF)[a]

How survivors felt they acquired Ebola	%	
	Men (n=22)	Women (n=38)
Washing body of EHF victim	0	21
Love touch	32	11
Transporting EHF patient to hospital	5	16
Caregiving of EHF patient	27	53

[a]More than one response possible per informant.

Traditional Healers

The term traditional healers is used here because it is commonly used by WHO and other international agencies. In the Gulu area, however, such healers are often referred to as witchdoctors. Both terms misrepresent the nature of what they do. The term traditional gives the impression that their practices have not changed since time immemorial, when, in fact, such healers are always changing their practices. For instance, as mentioned, they no longer suck out *yat* with their mouths because some healers who did so contracted and died from HIV/AIDS. Today such healers use a local sponge or type of grass to extract *yat*. The term witchdoctor is even more misleading because witches (called night dancers or *lajok*) are relatively uncommon in this area by comparison to the Bantu-speaking areas to the south, and few healers know how to treat witchcraft. Indigenous healer may be a more appropriate term.

Before this study was conducted, WHO and other international and national health workers felt that traditional healing practices of some healers led to the amplification of the outbreak. A female healer and some of the earliest EHF patients were often mentioned as examples. In September, a healer traveled from Gulu town to her rural village a few days after treating a known EHF patient. The healer became ill and reportedly treated patients by cutting and sucking poisons, such as *yat,* from ill patients, and thus infecting her patients with her bodily fluids. The healer died, and ≥10 deaths were subsequently associated with her healing. But village sources indicated that she did not treat people in the rural village and she did not have any of her healing tools (e.g., spear and rattle) because rebels in the rural areas kill healers caught with these implements (rebels view the work of healers as contrary to the ways of God). Rather, the healer infected many people because she was a prominent and powerful healer. Consequently, when she became ill in the rural area, many people assisted in her care, several different persons slept with her during the night to watch after her, and once she died, several persons assisted in the traditional washing of her body. This case occurred early in the outbreak, and misunderstanding led health authorities to ban all traditional healing. Traditional healers were stigmatized, which may have been unfortunate as all those we interviewed wanted to help in control efforts. As mentioned above, healers rarely cut the skin to remove *yat* or to insert medicines or herbs because of HIV/AIDS health education programs and the loss of several healers to that infection.

Stigmatization

Independent research on stigmatization was conducted by Kabananukye (9) so only a limited number of results from our study will be described. Adults were asked when they would feel comfortable touching a person who survived Ebola: on the day of hospital release, after 2 weeks, after 1 month, or after >1 month. The most common response (49%) was 1 month after hospital release. This response is consistent with the epidemic control protocol described previously, but many survivors experienced stigmatization long after this 1-month period, in part, because they continued to experience other illnesses (e.g., vision problems, fatigue, leg pains).

Many survivors experienced intense stigmatization. Some were not allowed to return home, many had all their good clothes burned, and some were abandoned by their spouses. Their children were told not to touch them, and wives were told to go back to their home villages. The discrimination also extended to family and village members. For instance, community members from one of the first rural villages affected were regularly turned away at the marketplace and watering hole. One man eventually committed suicide, in part, because he had lost his wife to EHF but also reportedly because of the stress of rejection, harassment, and discrimination in public because of his association with EHF. The survivors' questionnaires suggested that women experienced somewhat greater stigmatization than men. Table 3 summarizes some of these findings.

Table 3. Ways and locations in which Ebola survivors felt stigmatized

Locations in which survivors felt stigmatized	% of yes responses	
	Men (n=22)	Women (n=38)
Feared by others when you returned to the community	55	82
Rejected at market or store	36	58
Rejected at well or borehole	32	58
Rejected when walking through neighborhood	55	76

Discussion

Several limitations apply to our study: 1) it was conducted within a relatively short period of time (16 days); 2) researchers were not allowed to live in a village as participant observers because of political insecurity, and 3) the study was conducted at the end of the outbreak. Given these limitations, the study nevertheless provided useful data for control efforts.

Fred Dunn (10), a physician and anthropologist, developed a simple framework for integrating anthropologic work into disease control efforts. The framework is useful because it emphasizes identifying both health-enhancing and health-lowering beliefs and practices of both the local, national, and international communities. Many sociocultural studies tend to focus only on how local beliefs and practices amplify the disease (e.g., how traditional burial practices contribute to disease transmission); little attention is given to how local peoples' beliefs and practices might contribute to control efforts. Many models also do

not examine the beliefs and practices of the biomedical community. The data from this limited study are placed in Dunn's framework in Tables 4 and 5. These data indicate that local, national, and international actions contributed to the control of this outbreak. All of the health-lowering activities in the community were targeted for change by health educators. Most of the health-lowering activities of the national and international teams were recognized shortly after they occurred. Many beliefs and practices are neutral in that they do not help or hinder transmission of EHF. For instance, chasing away *gemo* by using *ryemo gemo* or *chani labolo* did not clearly help or hinder disease transmission.

Table 4. Community beliefs and practices that enhanced and lowered health of some persons during Gulu Ebola hemorrhagic fever outbreak

Health enhancing	Health lowering
Indigenous protocol for epidemics (see text)	Some aspects of burial and funeral practices: washing of body, dressing the body, love touches, and ritual washing of hands in common bowl of water
Elders sought to help organize the community	Transporting sick or dead by bike, cart, or other means
	Some aspects of traditional healing practices, such as cutting of body to insert medicines

Most national and international physicians, nurses, and healthcare workers are supportive of sociocultural studies, but most do not have the time, especially in outbreak situations, or tool kits to conduct the kinds of studies that might be useful. In the short term, social scientists can contribute to: 1) epidemiologic studies (how to identify persons, personal naming systems, kinship terms, clan names); 2) doctor-patient relations (international healthcare workers understanding of local explanatory models); 3) control efforts (cultural practices and beliefs that may be amplifying outbreak, identifying and mobilizing existing cultural institutions); and 4) health education (which cultural practices and beliefs to build upon, where to focus change).

Many national and international healthcare workers tend to view cultural practices and beliefs as something to overcome, and certain cultural burial practices (washing the body and love touches) did initially amplify EHF in Uganda. However, once people realized that EHF killed rapidly and classified it as *gemo*, a different set of cultural practices and beliefs were implemented. One reason the health education program worked so well was that it was in many ways consistent with indigenous epidemic control measures (isolation, suspension of greetings, dances, public funerals). Even the burying of victims at the airfield, while a bit dramatic for some, was consistent with burying *gemo* victims outside or at the edge of the village

Table 5. Beliefs and practices of the national and international healthcare professionals that enhanced and lowered health of some persons during Gulu Ebola hemorrhagic fever (EHF) outbreak

Health-enhancing beliefs and practices	Health-lowering beliefs and practices
Most national government health workers and decision makers spoke local language and had an understanding of local cultures	Unintended consequences of WHO[a] health education video: burning of houses of survivors
Establishment of isolation unit and use of barrier nursing	Taking bodies to burial ground before family members could verify the death. This practice led to sick persons hiding from family and health workers; family members being afraid to take sick persons to hospital; persons running away from the ambulance; and stories of Europeans selling body parts
Providing gloves and bleach to local communities	Omitting traditional healers from control efforts; they were ready and willing as a group to help mobilize the community
Medical care of Ebola victims including rehydration, control of vomiting, other drugs/medications	Early stages only: 1) nurses and healthcare nurses lacked training about barrier nursing, protective gear, and education about the transmission and nature of the disease; 2) lack of transport for sick patients; 3) international health workers not familiar with naming, kinship system, household organization of local communities
Multidimensional health education	Taking blood samples for research only or blood taken without reporting results back to persons or communities' increased distrust of healthcare workers
Suspension of the following activities: handshaking upon greeting, cutting by traditional healers, schools, discos, public funerals, traditional beer drinking	International team members conducting EHF studies for research only. This diverted time and energy from control efforts
Diagnostic laboratories for Ebola	
Ambulances to transport patients to hospital to isolate	
Reallocation of tasks of health workers to focus on EHF	
Use of mobile teams to follow all contacts and provide health education, support for survivors and impacted families	

[a]WHO, World Health Organization.

Sensitivity to cultural factors associated with the control of chronic infectious and parasitic disease has increased in the past 20 years, but little attention has been given to cultural factors associated with emerging infectious diseases, especially diseases such as EHF that cause rapid death. The urgent context of these outbreaks often

leads to the neglect of local people's feelings and knowledge. The general impression is that, without Western intervention, the epidemic would kill hundreds and spread to all parts of the world; local practices and beliefs are perceived only as amplifying the outbreaks. Our study was the first systematic sociocultural study of EHF. It showed that some cultural practices did indeed amplify the outbreak. However, an important finding was that local people have beliefs and practices in place that can be useful to control rapid epidemics, such as EHF, with high fatalities. Because local people have lived with high mortality rates and serious epidemics for some time, their knowledge may be useful to national and international teams in their efforts to control emerging diseases.

Acknowledgments

We thank the families and persons who openly shared their Ebola experiences; Cathy Roth, Thomas Oyok, Paul Onek, Okot Lokach, Claudio Blé, Dan Bausch, and Chris Lane for their comments and support; Karl Johnson and Barney Cline for their comments on earlier drafts of this paper; and Kitza Francis for assistance in administering the survivor questionnaire.

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1918 Influenza Pandemic Caused by Highly Conserved Viruses with Two Receptor-Binding Variants

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The Spanish influenza pandemic swept the globe in the autumn and winter of 1918-19, and resulted in the deaths of approximately 40 million people. Clinically, epidemiologically, and pathologically, the disease was remarkably uniform, which suggests that similar viruses were causing disease around the world. To assess the homogeneity of the 1918 pandemic influenza virus, partial hemagglutinin gene sequences have been determined for five cases, including two newly identified samples from London, United Kingdom. The strains show 98.9% to 99.8% nucleotide sequence identity. One of the few differences between the strains maps to the receptor-binding site of hemagglutinin, suggesting that two receptor-binding configurations were co-circulating during the pandemic. The results suggest that in the early stages of an influenza A pandemic, mutations that occur during replication do not become fixed so that a uniform "consensus" strain circulates for some time.

The 1918–19 influenza pandemic began, in some parts of the world, with mild outbreaks in the spring of 1918. In the fall of that year, a lethal wave swept the globe. Outbreaks occurred in early September in North America, Europe, and Africa and spread rapidly, so that the disease had peaked and declined worldwide by the end of December (1–4). Many areas had an additional wave of the disease in the early months of 1919. In most communities, the fall wave of the pandemic lasted approximately 1 month, with 25% to 30% of the population experiencing symptomatic disease. Clinically, epidemiologically, and pathologically, the disease was remarkably uniform, suggesting that similar viruses were causing disease worldwide (5). To assess the homogeneity of the 1918 pandemic influenza virus, partial hemagglutinin (HA) gene sequences were determined for strains from five cases, including two newly identified samples from London, United Kingdom. The strains show 98.9% to 99.8% nucleotide sequence identity. One of the few differences between the strains maps to the receptor-binding site of HA, which suggests that two receptor-binding configurations were co-circulating during the pandemic.

Influenza A virus is capable of rapid genetic change in mammals (6–8). Its polymerase complex lacks proofreading capability, such that one in five virus particles produced is likely to contain a change at one of its approximately 13,500 nt (9). If such a change provides the virus with a competitive advantage, that strain quickly replaces its predecessor. In humans, the need to escape preexisting immunity exerts positive selection pressure on changes in amino acids comprising the antigenic sites of the surface glycoproteins, HA and neuraminidase (NA) (6,10). The process of progressive change in the antigenic properties of the virus is called antigenic drift and results in the emergence of an antigenically distinct variant strain every 2–3 years. Between drift epidemics, the influenza virus appears to be antigenically uniform (11), but the degree of genetic uniformity has not been studied extensively.

In pandemic influenza, one or both of the virus's surface proteins are replaced with proteins to which the human population has no preexisting immunity (6,12). The virus then spreads explosively, producing symptomatic infection in up to one third of most populations. During the rapid initial spread of a pandemic strain, little antigenic pressure on the virus exists. One might expect the genetic structure under these circumstances to be relatively constant. However, the degree of genetic identity among viral isolates during a pandemic is not known. Very few full-length HA sequences of viruses from the peaks of the 1957 and 1968 pandemics are available, and all of these viruses had been grown at least once in eggs before sequencing—a process that can select for an unpredictable number of sequence changes (13,14). Therefore, this study represents an initial attempt to measure the degree of genetic homogeneity of a pandemic virus. Since the sequences have

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been obtained directly from clinical material, they contain no sequence changes attributable to culture.

Materials and Methods

Patients and Samples

The genetic sequences encoding the HA1 domains of three 1918 influenza strains have been determined (15,16). Two of the strains came from U.S. soldiers who died on September 26, 1918: one in Camp Upton, New York, and one in Fort Jackson, South Carolina. The third came from an Inuit woman who died in mid-November 1918 in a remote village on the Seward Peninsula of Alaska.

To obtain further samples for analysis, we examined autopsy material of 14 patients who died in the fall and winter of 1918 to 1919. The material consisted of formalin-fixed, paraffin-embedded tissues, stained slides, and clinical records from the files of the Morbid Anatomy Department of the Royal London Hospital. The cases were preselected by histologic criteria for further analysis, and samples were taken from patients who died from acute influenza after clinical courses of <1 week (16–18). Of these 14 lung samples, 4 were positive for influenza RNA on subsequent molecular genetic analysis, but only 2 had sufficient material for HA1 sequencing. The first patient was a 50-year-old woman admitted to the hospital on November 12, 1918, with influenza and pneumonia. She died on November 13. The postmortem diagnosis was bronchopneumonia. The second patient was a 25-year-old man admitted to the hospital on February 13, 1919. He died on February 15 of influenza. The postmortem diagnosis was lobar pneumonia with toxemia.

Methods

Sample preparation, reverse transcription, polymerase chain reaction (PCR), and sequencing were performed as described previously (15). (Primers used are available upon request.) PCR was performed from at least two separate reverse transcription reactions, and products from at least two PCR reactions were sequenced in each case to ensure accuracy and exclude amplification artifacts. Sequences used to evaluate the complexity of pandemic and epidemic influenza strains were obtained from the Influenza Sequence Database (available from: URL: http://www.flu.lanl.gov/).

Results

The 563-bp fragments sequenced for this study, encoding the antigenic and receptor-binding sites of the HA1 domain (19–21), represent the most variable portion of the influenza genome (Figure). The London cases were designated A/London/1/1918 (H1N1) and A/London/1/1919

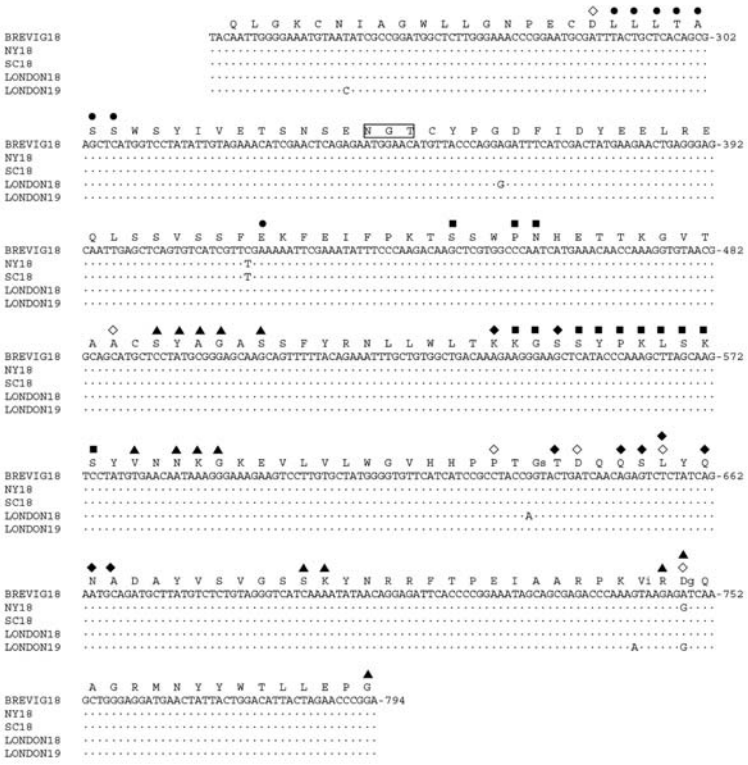

Figure. Partial HA1 domain cDNA sequences from five 1918-19 cases. A 563-bp fragment encoding antigenic (19,20) and receptor-binding (21) sites of the HA1 domain is shown, with the sequences aligned to A/Brevig Mission/1/1918 (BREVIG18) (15). Dots represent sequence identity as compared to BREVIG18. The numbering of the nucleotide sequence is aligned to A/PR/8/1934 (GenBank accession no. NC_002017) and refers to the sequence of the gene in the sense (mRNA) orientation. The partial HA1 translation product for BREVIG18 is shown above its cDNA sequence. Amino acid numbering is aligned to the H3 HA1 domain (15). Boxed amino acids indicate potential glycosylation sites as predicted by the sequence (15). Residues that have been shown experimentally to affect receptor-binding specificity in H1 HAs, D77, A138, P186, D190, L194, and D225 (21–23) are indicated by a ◇ symbol above these six residues. Residues defining four antigenic sites are indicated: Cb (●), Sa (■), Sb (◆), and Ca (▲) (19,20). Residues that have been mapped to both receptor-binding and antigenic sites (positions 194 and 225) are marked with two symbols. When a nucleotide change as compared to BREVIG18 results in a changed amino acid, the resultant amino acid is shown in lower case to the right of the BREVIG18 residue. Strain abbreviations and GenBank accession numbers: A/Brevig Mission/1/1918 (BREVIG18, # AF116575), A/South Carolina/1/1918 (SC18, # AF117241), A/New York/1/1918 (NY18, # AF116576), A/London/1/1918 (LONDON18, # AY184805), and A/London/1/1919 (LONDON19, # AY184806).

(H1N1). These two sequences, when compared to the three previously sequenced North American strains (15), differ from each other by 1 nt to 3 nt, showing a sequence identity of 98.9% to 99.8%.

A/London/1/1918 shows 2 nt differences, compared to A/Brevig Mission/1/1918, one of which would change the amino acid at codon 188 from G to S (amino acid numbering is aligned to the H3 influenza HA). This residue is near several of the residues that have been shown experimentally to affect receptor-binding specificity of H1 HAs (21–23) and next to one of the mapped Sb antigenic site residues (19,20). A/London/1/1919 shows 3 nt differences from A/Brevig Mission/1/1918, 2 of which are nonsynonymous, resulting in changes of V223I and D225G. The V223I change is near Ca antigenic site residues, and the D225G change is at a residue that functions both in receptor-binding and as a Ca antigenic site residue. Amino acid 225 also varies among North American strains; A/New York/1/1918, like A/London/1/1919, has a glycine at position 225, as do most avian influenza strains. A/South Carolina/1/1918 and A/Brevig Mission/1/1918, like A/London/1/1918 and most subsequent human H1 strains, have aspartic acid at this position (Figure) (15). The relative genetic homogeneity of the 1918–19 isolates encouraged us to analyze sequences from the 1957 and 1968 pandemics.

GenBank contains complete HA1 domain–encoding sequences for eight 1957 H2N2 strains. As noted in previous studies of receptor-binding specificity (22,24), the 1957 strains have undergone varying passage histories, but all have been passed at least once. Three of the strains have been sequenced more than once and differ by as many as 8 nt within the same strain. Between sequences, the number of nucleotide differences ranges from only 1 nt difference between A/Chile/6/1957 and A/Davis/1/1957 to 12 differences between one of the A/Japan/305/1957 sequences and one of the A/Singapore/1/1957 sequences. Overall, the sequences show 98.9% to 99.9% identity at the nucleotide level, and 98.5% to 100% identity at the amino acid level.

More limited sequence data are available for the 1968 H3N2 pandemic strains. The complete HA1 domain sequence is available for only three strains, two of which have been sequenced twice each. The two A/NT/60/68/29C sequences differ by 4 nt. The most divergent sequences differ by 24 nt (A/NT/60/68/29C vs. A/Hong Kong/1/68), thus showing 97.6% to 100% identity between sequences at the nucleotide level, and 96.0% to 100% identity at the amino acid level.

Studies from epidemic years have yielded similar results. A 2001 study (25) examined variation in the HA gene of human H3N2 viruses in Spain from 1996 to 2000. During this time, strains antigenically similar to A/Wuhan/359/1995 were replaced by strains similar to A/Sydney/5/1997 and then by strains similar to A/Panama/2007/1999. Within the groups of viruses belonging to each antigenic group, sequence variation was minimal. For example, among the viruses that reacted antigenically with Sydney, but not Panama and Wuhan, 2–10 nt differences occurred over the 591 nt sequenced (98.3% to 99.7% identity).

An unpublished study provides sequences of the HA1 domain of the H3-subtype HA of 16 A/Sydney/05/1997-like (H3N2) influenza virus isolates circulating in Canada during the 1997/98 influenza epidemic season (GenBank no. AF087700–AF087702, AF087707, AF087708, AF096306–AF096316) (26). Two of the isolates had identical sequences, while the others varied by 1 nt to 14 nt over 981 nt (98.6% to 100% identity).

Discussion

The three North American 1918 influenza strains sequenced previously were isolated from patients separated by nearly 2 months in time and almost 4,000 miles in distance (27). Two nucleotide differences were found among these three strains, one of which resulted in an amino acid substitution in the receptor-binding site (15). All three cases likely derived from the initial introduction of the fall wave into the United States, believed to have occurred in Boston in early September 1918. The virus then spread rapidly from Camp Devens, Massachusetts, the first U.S. army base to experience the epidemic, which then reached army bases throughout the eastern United States within 2 weeks (2). Influenza probably reached Brevig Mission, Alaska, via Seattle, Washington. The pandemic reached Camp Lewis, Washington, in mid-September, following the arrival of a troop ship from Philadelphia, Pennsylvania (1,2), and spread to Seattle by late September. After careful screening to exclude sick passengers, a ship left Seattle for Nome, Alaska, in mid-October, but days after its arrival local residents began falling ill (1). An account of the pandemic as it occurred in Brevig Mission reports that visitors from Nome brought the disease to the village in November (28). This chain of events suggests that the Alaskan outbreak was not the result of a separate introduction of the 1918 influenza from Asia to the West Coast of the United States.

The spring wave of the 1918 epidemic was widespread in France and Spain during April and May but did not reach England until June. The fall wave also arrived somewhat later in England than in continental Europe and the United States; peak mortality in London occurred during the first 2 weeks of November (2). A second peak occurred in the third week of February 1919. One strain from each of these peaks was sequenced for this study.

Our results show that strains separated by over 7,500 miles (Brevig Mission, Alaska, to London, United

Kingdom) and several months (September 26, 1918, to February 15, 1919) share a sequence identity of 99%. This level of genetic homogeneity is slightly higher than that seen for the available 1957 and 1968 pandemic strains, but the 1957 and 1968 strains were not sequenced directly from clinical material. Sequences from different passages of the same strain were sometimes as different from each other as they were from other strains (29), suggesting that sequence heterogeneity observed was the result of culture adaptation, making it impossible to determine how homogeneous the pandemic viruses actually were. Even so, the 1957 and 1968 pandemic strains show >97% identity between strains. Similar levels of genetic homogeneity were seen in strains from case-patients isolated from a drift epidemic in 1997. Thus, influenza viruses circulating during a single outbreak, whether epidemic or pandemic, show levels of sequence identity consistent with the uniformity of the 1918 cases.

Despite the uniformity of the 1918 strains, one of the variable sites is an amino acid known to be important in receptor binding (21). At a subset of amino acids critical for receptor binding, avian strains differ from swine H1s at only one amino acid, E190D (15). At these amino acids, two of the cases (A/New York/1/1918 and A/London/1/1919) are identical to that of A/sw/Iowa/1976/31 (a classical swine strain). The other 1918 cases have an additional change from the avian consensus at amino acid 225. Since swine viruses with the same receptor site as A/sw/Iowa/1976/31 bind both SAα2,3Gal and SAα2,6Gal (14), A/New York/1/1918 and A/London/1/1919 probably also had the capacity to bind both receptors. Because two of five 1918-19 analyzed fall wave strains from case-patients have the swine-like receptor-binding pattern, the E190D change alone is apparently sufficient to allow viral replication in the human respiratory tract. However, the existence of three strains with the additional G225D change shows that both receptor-binding variants were co-circulating throughout the pandemic. The current evidence does not suggest progression from one receptor-binding pattern to the other during the pandemic, since the two variants are present, on both continents, both early and late in the pandemic. Since residue 225 has also been identified as part of the Ca antigenic site (19), the co-circulating strains possibly differed in antigenic reactivity as well as receptor-binding characteristics.

This study is the first to examine the genetic homogeneity of a pandemic influenza virus directly from clinical material. The results suggest that in the early stages of a pandemic, mutations that occur during replication do not become fixed so that a uniform consensus strain circulates for some time. Studies of influenza strains circulating after 1919 should provide insight into how pandemic viruses evolve after the initial waves through immunologically vulnerable populations. In terms of pandemic planning, our results indicate that a specific antiviral drug or vaccine would have a uniform effect during the important and often lethal first wave of a pandemic (30,31).

This work was supported by a grant from the National Institute of Allergy and Infectious Diseases (R01 AI50619-01) to J.K.T. and by the intramural funds of the Armed Forces Institute of Pathology. J.S.O., C.L.B., and R.S.D. gratefully acknowledge financial support from the Wellcome Trust and the Ian Heap fund.

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Cephamycin Resistance in Clinical Isolates and Laboratory-derived Strains of *Escherichia coli*, Nova Scotia, Canada

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AmpC β-lactamase, altered porins, or both are usually responsible for cefoxitin resistance in *Escherichia coli*. We examined the relative importance of each. We studied 18 strains of clinical isolates with reduced cefoxitin susceptibility and 10 initially-susceptible strains passaged through cefoxitin-gradient plates. Of 18 wild-resistant strains, 9 had identical promoter mutations (including creation of a consensus 17-bp spacer) and related pulsed-field gel electrophoresis patterns; the other 9 strains were unrelated. Nine strains had attenuator mutations; two strains did not express OmpC or OmpF. After serial passage, 8 of 10 strains developed cefoxitin resistance, none developed promoter or attenuator mutations, 6 lost both the OmpC and OmpF porin proteins, and 1 showed decreased production of both. One strain had neither porin alteration or increased AmpC production. Porin mutants may occur more commonly and be less fit and less inclined to spread or cause disease than strains with increased β-lactamase expression.

The development of antibiotic resistance in *Escherichia coli* has important clinical implications. *E. coli* is among the most frequently isolated bacterium in a variety of clinical settings. The development of resistance to older agents such as ampicillin and trimethoprim-sulfamethoxazole, as well as the emerging problem of fluoroquinolone resistance, may substantially limit our antibiotic choices (1,2).

Although cephamycin-resistant *E. coli* is relatively uncommon, widespread use of β-lactam antiboties may contribute to the development and spread of these strains. In 1999, Sahm et al. reported that 0.16% of *E. coli* were resistant to cephamycins (3). At a local level, unpublished data from the Queen Elizabeth II Health Science Centre in Halifax, Nova Scotia indicated that, of the 5,767 strains of *E. coli* processed from urine samples, 0.4% were cephamycin resistant.

All strains of *E. coli* possess a gene that encodes an AmpC β-lactamase. Usually, almost no β-lactamase is produced because the gene is preceded by a weak promoter and a strong attenuator (4). Surveys of resistance mechanisms in cephamycin-resistant strains have most often identified promoter or attenuator mutations, which results in an up-regulation of AmpC β-lactamase production (5–7). Occasionally, cephamycin-resistant strains bear mobilized β-lactamases derived from bacteria such as *Citrobacter feundii* (8). In addition, mutation or altered expression of outer membrane proteins constituting porins can also contribute to cephamycin resistance. To our knowledge, no investigators have concurrently looked for alterations in porins in addition to promoter-attenuator mutations. Porin alterations might work together to produce a higher level of resistance. In addition, porin alterations may protect *E. coli* and allow subsequent selection for promoter and attenuator mutants.

We examined *E. coli* strains collected at our hospital to determine the basis for resistance. In addition, we created cephamycin-resistant strains of *E. coli* by serial passage on cefoxitin-containing medium to determine which of these two resistance mechanisms was predominant and if our findings were representative of those seen in clinical isolates.

Materials and Methods

Bacterial Strains

We collected strains of *E. coli* from midstream urine from inpatients and from patients in the community. Eighteen strains with reduced susceptibility (MIC ≥8 mg/L) to cefoxitin were included in the analysis, which represented all resistant strains collected during a 6-month period in 2001. For the in vitro development of resistance,

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we selected 10 clinical isolates from urine that were fully susceptible to β-lactam antibiotics. In both cases, we excluded duplicate strains from the same patient. *E. coli* isolates were identified with conventional biochemical reactions. Organisms were identified by spot indole and β-glucuronidase assays and confirmed by automated Vitek by using GNI+ cards, and antibiotic susceptibilities were performed by using GNS 606 cards (bioMerieux Canada Inc., St. Laurent, Quebec).

Analysis of Promoter and Attenuator Mutations

E. coli chromosomal DNA was isolated with a QIAmp DNA Mini Kit (Qiagen Inc., Valencia, CA), according to the manufacturer's instructions. Using standard methods, we performed polymerase chain reaction (PCR) with a previously published primer set and protocol which amplifies the region of DNA including the –35 box of the AmpC promoter and the 3′ end of the attenuator, producing a 271-bp amplicon (5). Amplification was performed in a PTC-200 Peltier Thermal Cycler (MJ Research, Boston, MA). The amplicons were resolved by 2% agarose gel electrophoresis and visualized after staining with ethidium bromide. The amplicons were purified by using the QIAquick PCR Purification Kit (Qiagen Inc.) and sequenced directly in both directions by using the dideoxy chain termination procedure of Sanger et al. on an ABI Prism automated sequencer at York University Core Molecular Laboratory, Toronto, Ontario, Canada.

Molecular Fingerprinting

Strains sharing similar promoter or attenuator mutations were fingerprinted by pulsed field gel electrophoresis (PFGE) by using a modification of the method of Gautom (9). In brief, a standardized suspension of *E. coli* was prepared from overnight cultures and treated with lysozyme and proteinase K. Plugs were prepared in low-melt agarose. Solidified plugs were deproteinated with sodium lauryl sarcosine and proteinase K, and then washed repeatedly. Two millimeter slices of plug were digested with *Xba*I at 37°C for 3 h in the recommended buffer. Plugs were loaded onto a 1% agarose gel and resolved with a CHEF-Mapper system (Bio-Rad Laboratories, Mississauga, Ontario).

Outer Membrane Profiles

Bacteria were grown overnight in Luria-Burtani (LB) broth with or without 4 mg/L of cefoxitin. To study Omp expression, 30 mL of LB broth was injected with 300 µL of a bacterial cell suspension from an overnight culture. Cultures were incubated at 37°C in a shaking water bath at 250 rpm to an optical density at 600 nm of 1.0. Cell membranes were disrupted with a sonicator for 2 min with 30-sec cycles intermittent on ice. Cell debris was removed by centrifugation at 10,000 *g* for 10 min at 4°C. Cytoplasmic membrane proteins were differentially solubilized for 20 min at room temperature with 1.7% sodium-lauryl-sarcosinate in 100 mM Tris, pH 8.0. The suspension was then centrifuged at 100,000 g for 20 min at 4°C, and the pellet containing the outer membrane proteins was resuspended in 100 µL sterile distilled water. Omp preparations were analyzed by urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 30 mA in gels prepared with 11% acrylamide, 0.3% bisacrylamide, 8 M urea, and 0.1% SDS using the discontinuous buffer system of Laemmli (10). The gels were stained with Coomassie brilliant blue. The positions of OmpC and OmpF on the Omp profiles were ascertained by comparing the profiles of Omp preparations from the *E. coli* reference strains MH760 (ompR472 OmpC- OmpF+) and MH1461 (envz11 OmpC+ OmpF-) (11).

Development of Cefoxitin-resistant Strains

Cefoxitin-resistant mutants were obtained by serially passaging the wild type strains on 9 cm x 9 cm^2 gradient plates containing a maximum of 32 mg/L cefoxitin in MH agar, as previously described (12). Plates were incubated at room temperature overnight before use to ensure proper diffusion of the antibiotic. Streaked plates were incubated overnight at 37°C and the colony that grew furthest up the cefoxitin gradient was selected and replated on a fresh gradient plate the following day. A total of 12 passages were performed for each strain at a maximum concentration of 32 mg/L cefoxitin and an additional 15 passages at a maximum of 128 mg/L cefoxitin. Isoelectric focusing was performed by using a modification of the method (13).

Results

Each of the 18 strains with reduced susceptibility to cefoxitin was also resistant to ampicillin, cephalothin, and amoxicillin/clavulanate acid. All were imipenem susceptible. Isoelectric focusing demonstrated chromosomal AmpC in all strains; no other β-lactamases were identified. A summary of promoter and attenuator mutations, as well as alterations in outer membrane profiles, is shown in Table 1 and Figures 1 and 2. Strains QE1–QE9 were identical or closely related by PFGE. Each strain had a 1-bp insertion in the spacer region between the –35 and –10 boxes. This insertion created a consensus 17-bp spacer. In addition to this mutation, these strains had additional mutations at –73, +6, and +81. Strain QE7 also had a deletion in the loop of the attenuator. None of these strains had changes in their outer membrane protein profiles.

The other nine strains (QE10–QE18) had different PFGE patterns and came from diverse locations (different hospitals and from both outpatients and inpatients). Strain QE10 had a C to T mutation at –42 and a G to A at –18,

Table 1. Summary of promoter/attenuator mutations and porin changes in 18 clinical strains of *Escherichia coli*, arranged by pulsed-field type[a]

PFGE type	Strain	MIC (µg/mL)			Patient Location	Promoter mutations	Attenuator mutations	Outer membrane protein (Omp) profile
		FOX	CAZ	CRO				
A	QE1	16	<8	<8	HCC	T insertion (–13) to consensus spacer (17 bp)	G to A, right stem; C to T, upstream of stem/loop	No abnormalities
A1	QE2	8	<8	<8	FD	T insertion (–13) to consensus spacer (17 bp)	C to T, upstream of stem/loop	No abnormalities
A2	QE3	>32	<8	<8	Inpatient Ward A	T insertion (–13) to consensus spacer (17 bp)	C to T, upstream of stem/loop; C deletion, right stem	No abnormalities
A2	QE4	16	<8	<8	FD	T insertion (–13) to consensus spacer (17 bp)	C to T, upstream of stem/loop	No abnormalities
A3	QE5	16	<8	<8	ER	T insertion (–13) to consensus spacer (17 bp)	C to T, upstream of stem/loop; C to T, left stem	No abnormalities
A4	QE6	>32	<8	<8	HCC	T insertion (–13) to consensus spacer (17 bp)	C to T, upstream of stem/loop	No abnormalities
A5	QE7	>32	<8	<8	HCC	T insertion (–13) to consensus spacer (17 bp)	C to T, upstream of stem/loop; ATG deletion in loop/right stem (+27 –29)	No abnormalities
A	QE8	16	<8	<8	FD	T insertion (–13) to consensus spacer (17 bp)	C to T, upstream of stem/loop; G to A, left stem	No abnormalities
A	QE9	>32	16	<8	Inpatient Ward B	T insertion (–13) to consensus spacer (17 bp)	C to T, upstream of stem/loop; G to A, right stem	No abnormalities
B	QE10	>32	<8	<8	Inpatient Ward C	C to T (–42); G to A (–18); C to T (–1)	C to A, left stem; C to T, downstream of stem/loop	No abnormalities
C	QE11	>32	<8	<8	Inpatient Ward C	G to A (–18); C to T (–1)	C to T, downstream of stem/loop	No abnormalities
D	QE12	>32	<8	<8	FD	G to A (–18); C to T (–1)	C to T, downstream stem/loop	Omp F-
E	QE13	>32	16	<8	FD	G to A in spacer (–28)	C to T, downstream stem/loop	No abnormalities
F	QE14	8	<8	<8	ER	T to A (–32), new –35 box; C to T (–11), new –10 box	None	No abnormalities
G	QE15	>32	16	<8	Inpatient Ward D	T to A (-32), new –35 box	None	Omp C-
H	QE16	>32	<8	<8	HCC	None	C to T, downstream stem/loop	No abnormalities
I	QE17	16	<8	<8	FD	None	C to T, downstream stem/loop	No abnormalities
J	QE18	>32	<8	<8	Inpatient Ward E	None	C deletion, right stem (+31); C to T, downstream stem/loop	No abnormalities

[a]PFGE, pulsed-field gel electrophoresis; FOX, cefoxitin; CAZ, ceftazidime; CRO, ceftriaxone; HCC, Hants Community Clinic; FD, family doctor's office; ER, emergency room.

which created a novel consensus –35. Two strains had the T to A mutation at –32 that is necessary to create the stronger –35 consensus sequence (QE14, 15). Strain QE14 also had the –11 C to T mutation that created the stronger –10 consensus sequence (TACAAT).

Only two strains had no promoter or attenuation loop mutations and no abnormalities of the outer membrane profile (QE16, QE17). Both of these strains had the C to T mutation at position +58 of the attenuator. This mutation would appear not to influence the development of the attenuation loop.

Of the 10 susceptible strains that were serially passaged on gradient plates, 8 developed resistance to cefoxitin (strains LD1–LD5, LD8–LD10). One of these strains had mutations in the AmpC promoter or attenuator regions (Table 2); this strain had two mutations, including a C to T mutation in the left stem of the attenuator, which would result in the transcription of a weak attenuation loop. Both of the mutations were also seen in the initial clinical isolates. This strain also had absent Omp C and Omp F. The remaining cefoxitin-resistant strains either lacked Omp C and Omp F (six strains) or had decreased amounts of OmpC and OmpF (one strain). One strain had no mutations in the promoter or attenuator and normal amounts of Omp C and Omp F.

Discussion

The emergence of *E. coli* strains resistant to extended-spectrum cephalosporins and cephamycins should be a cause of concern to clinicians managing infections in both

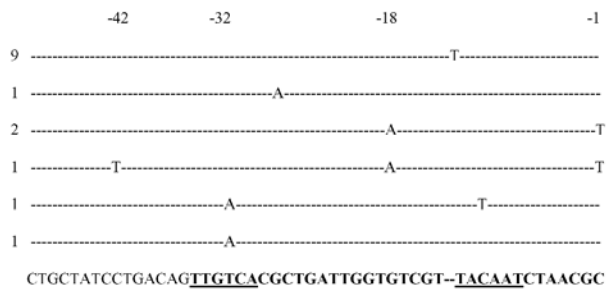

Figure 1. Sequences of *Escherichia coli* AmpC promoters showing mutations detected in cephamycin-resistant strains. The consensus sequence for E. coli K12 is shown in the last row. The promoter region is in boldface. The –35 and –10 (Pribnow box) hexamers are underlined. The number of strains with these mutations is indicated on the left.

the community and institutional setting. Extended-spectrum cephalosporins and penicillins combined with β- lactamase inhibitors are frequently used for both empirical and definitive treatment of *E. coli* infections. Strains resistant to cephamycins have emerged in recent years. Some of these strains have become resistant by virtue of their hyperproduction of chromosomally encoded AmpC β-lactamases (5–7). Others have acquired plasmidic β-lactamases; most often those derived from *Citrobacter freundii* (8).

In our survey of *E. coli* strains identified at our center, we found that no strains were resistant as a result of acquired-plasmid–mediated β-lactamases. These strains all produced substantial amounts of AmpC β-lactamase with the isoelectric point characteristic of the *E. coli*–derived AmpC. The predominant reason for the hyperproduction of AmpC appeared to be promoter mutations, attenuator mutations, or both.

Half of the strains appeared to be clonal in origin in that they had the same promoter mutation and the same attenuator mutations. Several of the strains had additional attenuator mutations; however, all of these strains had the same pulsed-field pattern. None of the strains had alterations in their OmpC or OmpF profiles. These strains came from diverse locations within the Queen Elizabeth II Health Sciences Centre complex and from other hospitals. Since we had limited information on the movement of the infected patients within the healthcare system, we are unable to define a common source.

The other nine strains had different pulsed-field patterns. Two strains each had similar promoter mutations, attenuator mutations, or both. Again, these strains came from different locations within our center, the community, and from referring hospitals. Two of these strains had altered outer membrane profiles; one lacked OmpF, the other OmpC. The promoter and attenuator mutations we detected were very remarkably similar to those that had been previously described in France, South Africa, Sweden, and Toronto, Canada. In particular the –42, –31, –18 mutations and spacer inserts have been consistently reported. The –11 promoter mutation that we observed was the second report of this mutation in the *E. coli* AmpC promoter (14).

We were able to develop resistance in 8 of 10 strains in vitro. Upon examination, seven of these strains had altered outer membrane protein profiles; six lacked both OmpC and OmpF, and one had decreased production of both of these porin proteins. One strain did not have promoter or attenuator mutations or alterations in the outer membrane protein profile. Sequencing of the promoters and attenuators indicated that only one had mutations that might have affected the amount of β-lactamase produced. However, this mutation was present in the wild strain as well.

We found that promoter abnormalities were common in clinical strains but not in lab-generated cefoxitin-resistant isolates. Increased resistance to antimicrobial agents has been shown to be associated with loss of porins in *E. coli* and other gram-negative organisms (15–19). The major porin proteins of *E. coli*, OmpF and OmpC, are differentially expressed at the transcriptional level by the two-component regulatory proteins, EnvZ and OmpR. The sensory protein, EnvZ, phosphorylates the transcriptional factor OmpR in response to environmental stress. The cellular level of the active form of OmpR, OmpR-phosphate (OmpR-P), is responsible for the differential expression of *ompF* and *ompC* (20). Therefore, the tension between the kinase and phosphatase activities of EnvZ controls the level of active OmpR-P in the cell. In the resistant strains selected on gradient plates, we found that most lacked both major outer membrane proteins in comparison to the respective parent strain. The lack of outer membrane protein expression in these isolates could occur by several

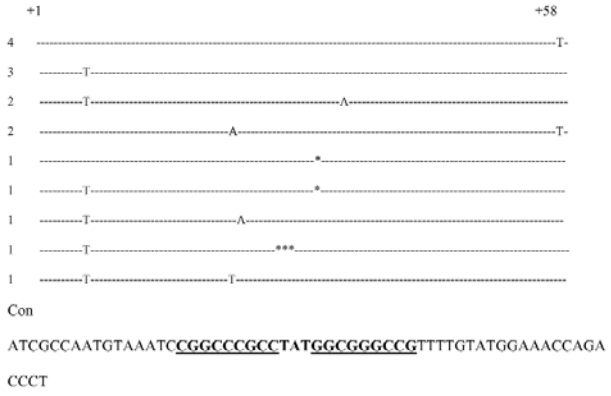

Figure 2. Sequences of *Escherichia coli* AmpC attenuators that were other than the consensus sequence for *E. coli* K12. The hairpin loop is in boldface, and the region of the dyad symmetry is underlined. * indicates bp deletion. The number of strains with these mutations is indicated on the left.

Table 2. Effect of serial passage on cefoxitin gradient on promoter and attenuator regions and outer membrane protein profiles of laboratory wild type *Escherichia coli*[a,b]

Strain	MIC before serial passage (μg/mL)	MIC after serial passage (μg/mL)	Outer membrane protein (Omp) profile
LD1	<2	>32	None
LD2	<2	>32	Omp C-, Omp F-
LD3	<2	16	Omp C-, Omp F-
LD4	<2	>32	Omp C-, Omp F-
LD5	<2	>32	Omp C-, Omp F-
LD6	<2	8	Not done
LD7	<2	4	Not done
LD8	<2	16	Omp C-, Omp F-
LD9	<2	>32	Omp C- , Omp F-
LD10	<2	>32	Decreased production of OmpC/F

[a]Only those mutations that arose following serial passage are shown. Some parental strains had mutations initially, which were retained in the mutants.
[b]No promoter/attenuator mutations affected β-lactamase production.

mechanisms, including loss of kinase activity of EnvZ and mutation of the transcription factor OmpR.

Alternatively, resistance may arise because of decreased pore diameter (21,22). The in vitro mutant without quantitative changes in Omp C or OmpF or promoter or attenuator mutations may have had this molecular lesion.

We postulate that, while porin deficient mutants are more readily selected by antimicrobial pressure, they are likely less fit. This finding is reflected by the fact that Omp changes are easily created in the laboratory but not found in clinical samples. As a result of their lower fitness, they are more likely to be replaced by wild *E. coli* when antimicrobial pressure has been removed. On the other hand, the widespread clonal dissemination of strains that hyperproduce AmpC by virtue of promoter or attenuator mutations suggests that they are much better able and more likely to contribute to the spread of cephamycin resistance.

The ampC β-lactamase produced by *E. coli* hydrolyzes penicillins, cephalosporins, and cephamycins. In doing so, β-lactamase increases the MICs to third-generation cephalosporins but, as our data suggest, seldom above the breakpoint set by the National Committee for Clinical Laboratory Standards (NCCLS). This situation is analogous to that of many TEM- and SHB-derived β-lactamases. Using third-generation cephalosporins or penicillin/β-lactamase–inhibitor combinations to treat serious infections caused by ampC up-regulated strains may be more imprudent. Just as we would not be inclined to treat an *E. coli* bearing an extended spectrum β-lactamase with a ceftriaxone MIC of 2 mg/L with ceftriaxone, we would not treat an ampC β-lactamase up-regulated *E. coli* strain with a third-generation cephalosporin. To the best of our knowledge, no publication has documented treatment failures in such circumstances, and no NCCLS guidelines exist. Nevertheless, our practice is to report all of these strains as resistant to third-generation cephalosporins and to cautioning against their use.

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This work was presented at the International Conference on Antimicrobial Agents and Chemotherapy (ICAAC) in San Diego, California, in September 2002.

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Mass Antibiotic Treatment for Group A Streptococcus Outbreaks in Two Long-Term Care Facilities[1]

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Outbreaks of invasive infections caused by group A β-hemolytic streptococcus (GAS) may occur in long-term care settings and are associated with a high case-fatality rate in debilitated adults. Targeting antibiotic treatment only to residents and staff known to be at specific risk of GAS may be an ineffective outbreak control measure. We describe two institutional outbreaks in which mass antibiotic treatment was used as a control measure. In the first instance, mass treatment was used after targeted antibiotic treatment was not successful. In the second instance, mass treatment was used to control a rapidly evolving outbreak with a high case-fatality rate. Although no further clinical cases were seen after the introduction of mass antibiotic treatment, persistence of the outbreak strain was documented in one institution >1 year after cases had ceased. Strain persistence was associated with the presence of a chronically colonized resident and poor infection control practices.

Group A β-hemolytic streptococcus (GAS) has a long-standing association with pharyngitis, skin and soft tissue infection, and pneumonia (1–3). In the past decade, reports of GAS-associated necrotizing fasciitis and streptococcal toxic shock syndrome (TSS) have increased (2,4). Increasingly, outbreaks of invasive GAS are recognized in long-term care facilities (5–10). In the Canadian province of Ontario, 4% of invasive GAS cases occur in long-term care facilities, and of these cases, one third are outbreak associated (5). In long-term care settings, a high case-fatality rate has been observed (40% to 60%) (5,11,12).

Guidelines for control of GAS outbreaks in nursing homes were developed by the Ontario Ministry of Health in 1995 (13). These guidelines emphasize the identification and elimination of colonization through providing antibiotics to those known to be carriers of the bacterium. Although such an approach should minimize antibiotic exposure, carrying it out in practice could be difficult, as cultures may be falsely negative (14), and persons could become colonized or transmit colonization to others in the interval between culturing and treatment. Antibiotic treatment of all residents and staff would not be subject to such limitations. We present two outbreaks of invasive GAS infection in long-term care facilities in Hamilton, Ontario, Canada. Each was caused by transmission of a single well-characterized outbreak strain, and in both cases, mass antibiotic treatment was used to control the outbreak.

Methods

Epidemiologic Investigations

In the province of Ontario, invasive infections with GAS (isolation of GAS from blood or an otherwise sterile site) and cases of suspected TSS and necrotizing fasciitis must be reported to local public health authorities (15). Reports of invasive GAS infection originating from long-term care institutions in the city of Hamilton resulted in an epidemiologic investigation by the City of Hamilton Social and Public Health Services Department. In the two outbreaks described below, investigations consisted of site visits with line-listings created to identify facility residents and staff; reviews of recent admissions, discharges, and deaths among residents; and reviews of reports of illness among residents and staff. Environmental investigations and reviews of infection control practices were also performed.

Attempts were made to identify facility residents and staff with close epidemiologic linkage to the index patients

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[1]Presented in part at the 12th Annual Scientific Meeting of the Society for Healthcare Epidemiology of America (SHEA), April 6–9, 2002, Salt Lake City, Utah.

(e.g., caregivers, roommates, and close friends). Although typically only persons with epidemiologic linkage to index patients are cultured for possible GAS carriage, the circumstances described below resulted in more widespread collection of culture specimens. Specimens were obtained from noses and pharynges of residents and staff, and attempts were made to obtain cultures of perirectal areas and wounds in facility residents. In the second outbreak described below, staff provided self-collected rectal and vaginal swabs.

Microbiologic Evaluation and Characterization of GAS Strains

Swabs from institution residents and staff were plated onto blood agar, with group A streptococcus identified by β-hemolysis and Lancefield typing using standard commercially available latex agglutination methods, as described elsewhere (16). Isolates were subsequently evaluated for relatedness by using pulsed-field gel electrophoresis (PFGE). Briefly, PFGE was performed using fresh overnight cultures of GAS strains. Isolates were suspended in buffered saline, and solutions were adjusted in volume until optical densities (OD) were identical (1.6 OD at 610 nm). To prepare genomic DNA, 500 mL of cell suspension from each strain was mixed with an equal volume of 2% low melting agarose solution. The agarose plug was prepared by using a commercially available plug mould (Bio-Rad Canada Limited, Mississauga, Ontario, Canada). All GAS plugs were treated in lysis buffer containing 50 mg/mL lysozyme at 37°C overnight, extensively washed in washing buffer, and then treated in a solution containing 50 μg/mL protease K at 55°C overnight. All plugs were extensively washed before being restricted by Sma-I enzyme. PFGE was run by using a 1% agarose gel at 6 volts/cm; run time was 26 h with an initial switch time of 20 s and a final switch time of 60 s. Following gel electrophoresis, the gel was stained in a solution containing 0.5 μg/mL ethidium bromide and photographed by using the Bio-Rad Multi-analysis CCD camera system. ATCC strain 19615 was included as the quality control strain.

M and T serologic typing and opacity factor determination were performed on all isolates with standardized methods (17). In addition, antiopacity factor (AOF) typing was performed on all isolates that were opacity factor-positive (17). All antisera were prepared in-house. Because of the difficulty of producing antisera for some M types, AOF typing has been frequently used to predict the M type (18,19). Although AOF typing does not always have the same type specificity as M typing, this approach is considered valid for most strains from developed countries (20). AOF typing was used to predict the following M types: 9, 11, 25, 28, 48, 77, 78 and 92 (PT5118).

Results

Outbreak 1

In October 2000, the Hamilton public health unit was notified of a 97-year-old female nursing home resident (index case-patient 1) admitted to an area hospital with cellulitis and group A streptococcal bacteremia. The nursing home was a 386-bed facility in a suburban area. Cultures were obtained as described from 87 residents and staff who might have had contact with the person with invasive disease. One resident had a throat culture positive for group A streptococcus. This isolate was genetically indistinguishable by PFGE analysis from the invasive isolate obtained from index case-patient 1, and both strains were typed as M77 T9/10, and positive for serum opacity factor.

Intervention consisted of reinforcement of standard infection control practices with facility staff and treatment of the person with pharyngeal colonization with a 10-day course of cephalexin, 250 mg four times per day. Follow-up cultures from this person taken 1 month after completion of treatment were negative. Index case-patient 1 died during the course of the investigation of apparently unrelated cardiac disease.

In January 2001, an 87-year-old female resident of the same long-term care facility was transported to hospital with fever (index case-patient 2), and GAS was isolated from blood cultures drawn at admission. Several days later, GAS was isolated from the wound of another facility resident. Screening cultures performed on 195 residents and staff with a history of contact with one of these two infected persons indicated that seven residents and one staff member were colonized with GAS. PFGE showed that the strains from all resident carriers were identical to the wound and blood isolates, and to the isolate obtained from index case 1. The bacterial isolate obtained from the staff member was determined by PFGE to be unrelated to the outbreak strain. Colonized residents and staff were treated with cephalexin as had been done with the previous patient. Cultures obtained from treated persons 1 month after completion of therapy were again negative.

In May 2001, another resident of this nursing home was transported to hospital with GAS bacteremia (index case-patient 3). At this time, all 386 residents and 135 staff of the facility were screened for GAS carriage by culturing nasopharynx specimens. Eleven residents and three staff members were identified as carrying group A streptococcus. Thirteen of these (11 residents and two staff members) subsequently proved to be carrying strains identified by PFGE analysis to be identical to the strain obtained from index case-patient 2 (Figure 1) (prevalence of colonization or infection among residents was 2.8% vs. prevalence of colonization 1.5% among staff , p=0.38 by chi-square test).

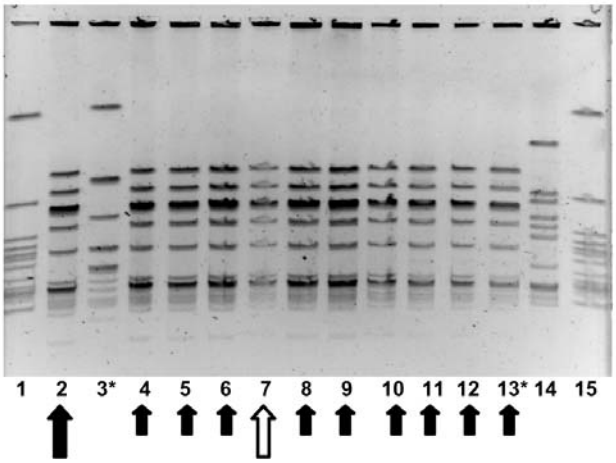

Figure 1. Molecular epidemiology of group A streptococcus (GAS) strains in outbreak 1. Pulsed-field gel electrophoresis, demonstrating relatedness of group A streptococcal isolates from an person with clinical illness from GAS, a person with chronic colonization with GAS, and asymptomatically colonized facility staff and residents. Lanes 1 and 15 contain an ATCC quality control strain. Lane 14 contains an isolate from another nursing facility, unrelated to outbreak 1. The isolate in lane 2 (large solid arrow) was obtained from index case-patient 2 in January 2001. The isolate in lane 7 (large hollow arrow) was obtained from a person with chronic GAS colonization (resident A) in May 2001. Small solid arrows denote electrophoretically identical GAS strains from other persons with asymptomatic colonization with group A streptococcus in May 2001. Asterisk denotes staff member.

One of the residents colonized with GAS (resident A) was a 69-year-old woman, immobilized by severe neurologic disease, who had a suprapubic bladder catheter and a gastrostomy tube. Resident A was subsequently determined to have had urine and wound cultures positive for GAS in July 2000, 3 months before index case-patient 1 sought treatment; these isolates were unavailable for evaluation. After consultation with local communicable disease control experts, the public health team recommended administering antibiotics to all facility residents and staff. All residents were treated with a 10-day course of cephalexin or penicillin VK, with erythromycin given to persons allergic to β-lactam agents. Similar antibiotic regimens were recommended for all staff members. No data are available with regard to the degree of compliance with antibiotic therapy among staff. However, all previously colonized staff members were culture-negative for group A streptococcus on repeat culturing 1 month later.

Ten of 11 treated residents had cultures negative for group A streptococcus 1 month after treatment. Resident A remained persistently colonized at gastrostomy and catheter sites, despite a subsequent prolonged course of oral clindamycin. This resident was placed on contact isolation precautions, with staff using gowns and gloves when participating in her care. She died in July 2002.

No subsequent cases of invasive group A streptococcal disease have been reported at this facility as of February 2003. However, in August 2002, GAS was identified in cultures of eye drainage and wound drainage from three residents of a single wing of the facility (Figure 2). PFGE demonstrated two of the three isolates to be indistinguishable by PFGE from isolates obtained from resident A. Neither of these persons had been present in the facility at the time of invasive cases, and neither had been housed in the same part of the facility as resident A. An audit of infection control procedures at that time showed several violations of standard infection control measures, including the reuse of a single washcloth on multiple residents of the facility.

Outbreak 2

In November 2001, the Hamilton public health unit was notified of two deaths from invasive group A streptococcal disease at a 126-bed long-term care facility. The first case-patient was a 78-year-old man who had been admitted to the hospital with fever and possible pneumonia 2 days before. A blood culture grew GAS. The second case-patient was a 61-year-old man with Parkinson's disease and dementia who had been admitted to the hospital 1 day before with soft skin tissue infection and likely necrotizing fasciitis. GAS was subsequently isolated from blood cultures. Both patients died on the day the cases were reported to the public health department.

A site visit was performed. The facility had 127 residents and 150 staff members. The two cases had originat-

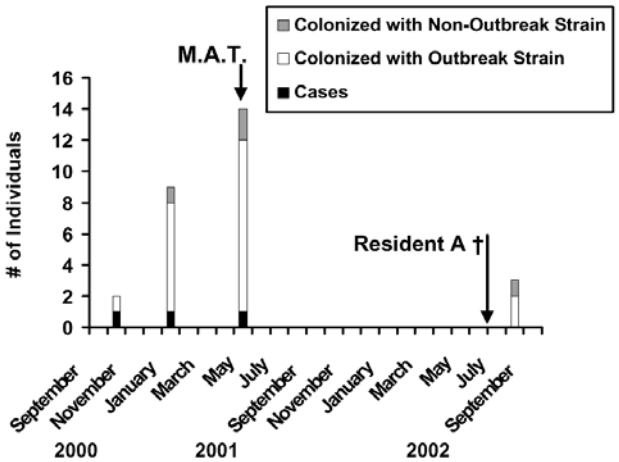

Figure 2. Epidemic curve for outbreak 1. Clinical cases (black bars) of invasive GAS infection occurred at intervals of 3 to 4 months. With the occurrence of cases, acquisition of culture specimens resulted in identification of asymptomatic colonization with the outbreak strain (white bars) or unrelated strains (hatched bars) in other residents and staff. No additional clinical cases occurred after mass antibiotic treatment (M.A.T.); resident A died (†) in July 2002; colonization of two residents with the outbreak strain was recognized 1 month later.

ed on different floors of the facility (2nd and 4th). A large holiday party had been held in the communal dining hall 4 days previously, providing an opportunity for residents, staff, and community members to mingle. In addition, a healthcare worker reportedly had pharyngitis, a rash, and desquamation of the palms and had received a diagnosis of scarlet fever 3 weeks earlier.

Due to the lack of any epidemiologic link between case-patients and the population mixing that had occurred at the holiday party, swab cultures were collected on all facility residents and all available staff members. Epidemic control measures were introduced and included restriction of new admissions to the facility and reinforcement of infection control practices. Based on the rapidity of events, the apparent high case-fatality rate, and recent experience with the failure of targeted culturing and treatment (as described in outbreak 1), a decision was made to initiate mass antibiotic treatment for all facility residents and staff. After that decision, but before mass antibiotic treatment could be initiated, an 89-year-old male resident was admitted to the hospital with congestive heart failure. Gram stain of sputum indicated gram-positive cocci in chains. This man died 36 h after admission; GAS was isolated from blood cultures taken at hospital admission (Figure 3).

Mass antibiotic treatment was initiated approximately 48 h after the start of the investigation, with azithromycin, 250 mg orally each day, administered for 5 days to residents and to staff who wished to be treated at the facility. Staff who wished to receive antibiotic treatment from their personal physicians were permitted to do so. Subsequently, GAS was isolated from nasal and pharyngeal cultures of two staff members and five residents of the facility. GAS was also isolated from a wound culture from a facility resident.

All colonized residents were negative for GAS on repeat cultures, 4 weeks after receiving antibiotics. One staff member remained culture positive 2 weeks after completing a course of cephalexin provided by her personal physician; she received two additional courses of β-lactam antibiotics and was documented to be culture negative for GAS 4 weeks after she completed the second antibiotic course.

PFGE performed on the three GAS strains from case-patients and the seven strains from colonized residents and staff showed that four colonized persons had isolates identical to those from the deceased case-patients; all were serotype M1 T1 and were serum opacity factor negative (Figure 4). The remaining three isolates represented two distinct strains unrelated to the outbreak strain. The proportion of residents colonized or infected with the outbreak strain was significantly higher than the proportion of staff colonized (4.7% vs. 0.7%, p=0.03 by chi-square test).

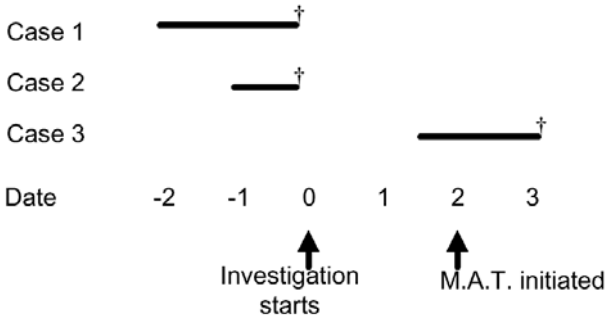

Figure 3. Timeline for outbreak 2. Solid lines represent the time of onset and duration of illness among three cases with invasive GAS infection in outbreak 2, relative to the initiation of the outbreak investigation (date=0). Daggers (†) denote death. Mass antibiotic treatment (M.A.T.) was started 2 days after the investigation was initiated.

Discussion

Our description of outbreaks of multiple cases of invasive disease attributable to a single, identified strain of GAS in long-term care institutions is consistent with prior reports (5-10). Outbreak 1 was caused by an M77 strain, an uncommon cause of invasive streptococcal disease in Canada (21). The second outbreak was caused by an M1-type strain, the most common isolate type in Canada, which may be more virulent than other serotypes (21,22).

Epidemiologic investigation of these two outbreaks showed a higher prevalence of colonization with the out-

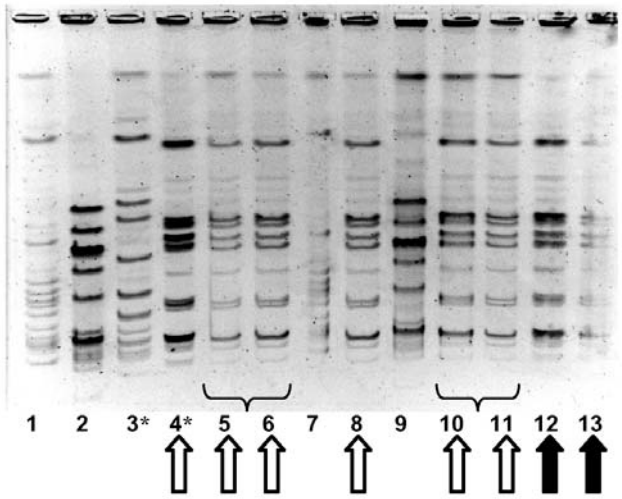

Figure 4. Molecular epidemiology of group A streptococcal strains in outbreak 2. Pulsed-field gel electrophoresis, demonstrating relatedness of group A streptococcal isolates from facility staff and residents. Lanes 1 and 7 contain an ATCC quality control strain. Solid arrows denote identical strains from two of the three persons in whom fatal invasive group A streptococcal infection developed; the third person with invasive disease had an electrophoretically identical strain (not shown). Hollow arrows denote identical strains from persons with asymptomatic colonization with group A streptococcus. Brackets denote duplicate strains from the same person; asterisk denotes staff member.

break strains among residents than among staff, although this difference was not statistically significant in outbreak 1. Given the limited mobility of the residents of both facilities, such a difference would be consistent with disease transmission involving asymptomatically colonized healthcare workers. Transmission may also have occurred as a result of transient carriage of the organism by healthcare workers, as may occur with nosocomial transmission of *Clostridium difficile* and vancomycin-resistant enterococcus (23,24), or as a result of contaminated fomites, such the washcloth described in outbreak 1. Other possible mechanisms of transmission include direct resident-to-resident transmission in the context of such social events as the holiday party described in outbreak 2 and foodborne transmission (25).

Any of these mechanisms of spread could limit the expected effectiveness of screening and targeted antibiotic treatment, as culturing of only residents and staff with a clear epidemiologic link to cases would not be expected to identify all colonized persons. This situation was well demonstrated in outbreak 1, which involved probable worker transmission of a strain from an immobile, chronically colonized person (resident A) to other residents in this institution. Although likely important in the perpetuation of the outbreak, resident A was identified incidentally when cultures were performed on all facility residents 8 months after the initial case of invasive disease was reported. The importance of silent carriage of GAS in the perpetuation of outbreaks has been well-described in both healthcare (26,27) and community (28) contexts.

Our use of mass antibiotic treatment as an epidemic control measure appears to have been successful, inasmuch as no further cases of invasive GAS disease have been reported from either facility at the time of writing (February 2003). However, the documented persistence of the bacterial strain implicated in outbreak 1 after the death of resident A suggests that breaches in infection control practices (as identified during site visits), combined with the presence of a persistently colonized resident or staff person, may limit the effectiveness of this control measure. Such a control measure would also not prevent the repeated reimportation of a circulating community strain of GAS into a facility by healthcare workers or visiting family members (29,30).

The impact of wide-scale antibiotic use on pathogenic microorganisms in long-term care institutions also remains an issue of concern. Resistance to macrolides among GAS isolates appears to be increasing in frequency (31,32), and the overuse of β-lactam antibiotics and macrolides may adversely affect the antibiotic susceptibility patterns of such common pathogens as *Streptococcus pneumoniae* (33–36).

These two outbreaks of invasive GAS in the long-term care setting highlight the limitations of targeted culturing and antibiotic treatment as an outbreak control strategy. However, our experience also highlights the limitations of mass treatment as a control strategy, particularly when poor infection control practices persist and chronically colonized residents are present. Further research into the epidemiology and transmission of GAS in the long-term care setting will help refine the optimal approach to managing these challenging outbreaks.

Acknowledgments

We thank the staff of the Health Protection Branch, City of Hamilton Social and Public Health Services Department, for their efforts in the investigation and control of the two outbreaks described; Mark Loeb, Allison McGeer, Ross Pennie, and Fiona Smaill for their helpful advice; and Lydia Napper for expert assistance in manuscript preparation.

Dr. Fisman was supported by the Ontario Public Health Research, Education, and Development Program.

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Anthelmintic Baiting of Foxes against Urban Contamination with *Echinococcus multilocularis*

Daniel Hegglin,* Paul I. Ward,* and Peter Deplazes*

In recent years, increases in the urban fox population have been observed in many countries of the Northern Hemisphere. As a result, Echinococcus multilocularis has entered the urban environment. Because of a possible increased risk for alveolar echinococcosis, intervention strategies need to be evaluated. In Zürich, Switzerland, 50 praziquantel-containing baits per km^2 were distributed monthly in six 1-km^2 bait areas and one 6-km^2 bait area from April 2000 through October 2001. The proportion of *E. multilocularis* coproantigen–positive fox fecal samples collected remained unchanged in six control areas but decreased significantly in the 1-km^2 bait areas (from 38.6% to 5.5%) and in the 6-km^2 bait area (from 66.7% to 1.8%). *E. multilocularis* prevalence in the intermediate host *Arvicola terrestris* also decreased significantly in baited areas. This controlled baiting study shows that a pronounced reduction of *E. multilocularis* egg contamination is feasible in urban areas where the organism is highly endemic.

The zoonotic tapeworm *Echinococcus multilocularis* is typically perpetuated in a wild life cycle, which includes foxes (genera *Vulpes* and *Alopex*) as definitive hosts and various rodent species as intermediate hosts (1). In addition, eggs are accidentally ingested by humans; the metacestodes enter mainly the liver and cause alveolar echinococcosis, a severe, sometimes fatal disease if left untreated (2,3).

Few studies have been performed on the epidemiology of alveolar echinococcosis. Risk factors for alveolar echinococcosis may include occupational and behavioral activities. However, hunters, trappers, and persons working with fur were not at increased risk for alveolar echinococcosis in South Dakota (4). Data from Europe have indicated that farming activities increase the risk for infection (5,6). Contamination of the rural environment with *E. multilocularis* connected with farming activities was indirectly demonstrated by high prevalences of alveolar echinococcosis in sows kept indoors but fed with grass (7). Areas with high water vole (*Arvicola terrestris*) densities yielded a 10-fold higher risk for human alveolar echinococcosis compared with areas with low densities of this important intermediate host (8). In an area where the organism is highly endemic, up to 39% of *A. terrestris* and up to 7% of dogs with free access to rodents were infected with *E. multilocularis* (9), and persons who have kept dogs around dwellings were at higher risk for alveolar echinococcosis on St. Lawrence Island, Alaska (10).

Red foxes (*Vulpes vulpes*) are likely to be the most important final host in many regions (11). In the past 2 decades, foxes have started to colonize in cities around the world (12–14), and evidence of the parasite cycle in urban areas is increasing (13,15,16). In Zürich, Switzerland, one study found that 47% of the urban fox population was infected with *E. multilocularis* (17).

The high number of infected foxes in cities and villages, in close contact with domestic pets and humans, could increase the risk of alveolar echinococcosis (16). The disease has an incubation period of 5 to 15 years; therefore, whether the actual incidence rate of alveolar echinococcosis reflects a continuing stable and low infection risk or whether the increased infection pressure in highly populated areas will lead to a delayed increase in the incidence of alveolar echinococcosis cases in the future is unclear (3). However, ecologic changes resulted in a very high alveolar echinococcosis prevalence of 4.0% in China, which is highly endemic for the organism (18). The high prevalence of *E. multilocularis* in densely populated areas and the increase of foxes living in close vicinity to humans strongly suggest that evaluating possible intervention strategies is prudent.

Few field studies focus on anthelmintic treatment of definitive hosts. Rausch et al. (10) demonstrated in a village that was hyperendemic for the organism (St. Lawrence Island, Alaska) that continual treatment of dogs with praziquantel reduces infection pressure of *E. multilocularis*, resulting in lower prevalence in locally trapped

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voles. In extended rural areas of Germany and Japan, praziquantel baits lowered the prevalence of *E. multilocularis* in foxes (19–21). These results cannot be transferred to the condition of agglomerations and urban areas, where until now no attempt has been made to evaluate an intervention strategy for foxes.

The urban cycle of *E. multilocularis* was studied intensively in Zürich, Switzerland (16,17,22). Analyses of fox stomachs indicated that *A. terrestris* was the most frequently consumed intermediate host (23), and *E. multilocularis* is highly prevalent (mean 9.1%, maximum 20.9%) in this vole species, which lives predominantly along the city border (22). Accordingly, the prevalence of *E. multilocularis* in foxes was significantly higher in the urban periphery than in more central areas (17), and the infection risk for alveolar echinococcosis might therefore be concentrated mainly in delimited areas in the urban periphery (16). Since urban inhabitants frequently use the zones of highest contamination for recreational activities and their domestic cats and dogs have access to infected voles, the urban periphery may represent a risk for alveolar echinococcosis.

In this controlled experimental field study, we investigated the effect of anthelmintic baiting in defined urban areas where the organism is highly endemic and tested whether *E. multilocularis* egg contamination was significantly reduced. We also examined whether, as an expected consequence, its prevalence in urban intermediate hosts diminished.

Methods

Study Area

The study was conducted in the community of Zürich and its surroundings. Zürich covers 92 km^2 and has a population of 360,000. Fifty-three percent of Zürich is an urban area with industrial, commercial, and residential buildings; the other portion of Zürich is 24% forest, 17% agricultural, and 6% water. We divided this area into zones: urban, border, and periurban zone. The urban zone is mainly residential with little green space. The periurban zone consists of forests, fields, pastures, and meadows. The border zone, which divides the urban and the periurban zone, was defined as extending 250 m from the border of the urban area with buildings into the residential area of the city and 250 m into the periurban surroundings. This zone includes mostly residential areas, allotments, cemeteries, sports fields, public places, and pastures. The border zone and the periurban zone are used by the public for recreational activities.

As far as hunting is concerned, Zürich is organized as a game sanctuary and, compared to the high population density of >10 adult foxes per km^2 (24), the hunting bag (foxes shot by game wardens) was relatively low during the course of this study (1.0 shot foxes per km^2 and year).

Baits

Commercial baits were used in the study (Impfstoffwerk Dessau Tornau GmbH, Rosslau, Germany). Each weighed 13.5 g, and the matrix consisted of Altrofox 91 (Impfstoffwerk Dessau Tornau GmbH). This matrix is the same one as in the widely used rabies vaccine bait Rabifox (Impfstoffwerk Dessau Tornau GmbH). The baits contained 50 mg of the anthelmintic praziquantel (Droncit Bayer AG, Leverkusen, Germany), a highly efficient drug against adult cestodes.

Experiment Design

Along the urban periphery we selected six bait and six control areas of 1 km^2 each and an additional bait area of 6 km^2. Bait and control areas were separated by at least 600 m to minimize the chance of foxes using two areas (Figure 1). All areas included a similar amount of urban area with buildings, open spaces (public parks, cemeteries, allotment gardens, and meadows), and woodlands in a pattern typical for the urban fringe (Figure 1). In baited areas, 50 praziquantel-containing baits per km^2 were distributed monthly (intervals of 25 to 35 days) during 19 months from April 2000 through October 2001. Baits were distributed manually at places that were most likely to be frequented by foxes (e.g., where fox tracks had been seen, fox dens, and compost heaps) but not by dogs. To avoid olfactory contamination, baits were always handled with rubber gloves. Baits were covered with surrounding material to protect them from sun at exposed sites.

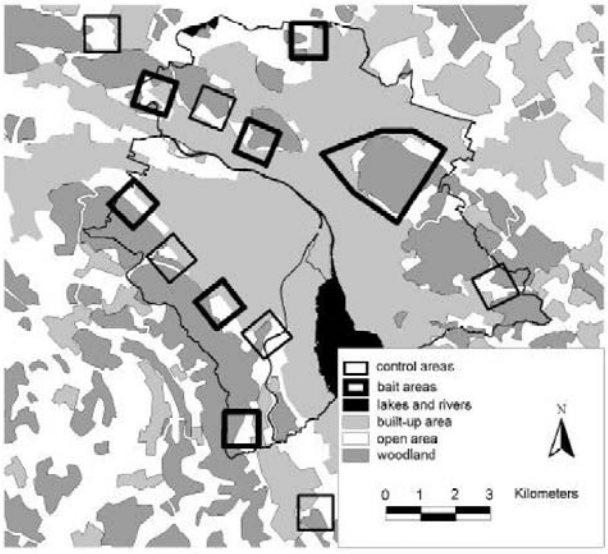

Figure 1. Study area of the controlled anthelmintic baiting experiment in the conurbation of the city of Zürich. 50 Praziquantel-containing baits per km^2 were delivered monthly in six 1-km^2 bait areas and one 6-km^2 bait area, that alternated along the urban fringe with six control areas. Black line, Zürich border.

Sampling and Analyses of Fox Fecal Samples

Fox fecal samples were collected at least once per month in bait and control areas and their immediate vicinity during the following periods: winter 1999/2000 (November 1999 to February 2000), spring 2000 (April to June 2000), summer/autumn 2000 (July to October 2000), winter 2000/01 (November 2000 to February 2001), and summer/autumn 2001 (July to October 2001). Several criteria, such as size, shape, homogeneity, and smell of the droppings, were used to distinguish fox fecal samples from other fecal samples (22). For each of the 1,537 collected fecal samples, we recorded the exact position to an accuracy of 20 m.

E. multilocularis coproantigen was detected by a sandwich–enzyme-linked immunosorbent assay (EM–ELISA) (25), which was recently validated for testing field fecal samples in eastern France (26) and our study area (22). Coproantigen-positive fecal samples, collected in bait and control areas during 2001, were further evaluated to check whether infected foxes in bait areas had predominantly fresh, prepatent infections and did not excrete *E. multilocularis* eggs. Therefore, we isolated taeniid eggs from the fecal samples followed by *E. multilocularis*–specific polymerase chain reaction (PCR) as described previously (27).

Sampling and Analyses of *A. terrestris*

In Zürich, we found the highest prevalence of *E. multilocularis* coproantigen in the intermediate host *A. terrestris* (22). Therefore, we focused on this species to evaluate the effect of bait distribution on intermediate host populations. *A. terrestris* were trapped with unbaited tong traps (Hauptner Instrumente GmbH, Dietlikon, Switzerland) and Topcat traps (TOPCAT GmbH, Wintersingen, Switzerland). Traps were set in intervals of 1 to 2 months in each bait and control area from April to November 2000 and from July to October 2001. Additionally, in the 6–km^2 bait area, traps were regularly set from July 1999 to February 2000. All 1,229 dissected rodents were carefully examined macroscopically for lesions in their livers and other organs. Lesions ≥2 mm in diameter were investigated for *E. multilocularis* metacestode tissue either by examining morphologic features or by DNA detection by using modified PCR (28).

Statistical Analysis

Statistical analyses were performed with SPSS–PC version 10.0. Stepwise backward logistic regression was used to test the effect of baiting on the proportion of coproantigen-positive fecal samples and on its prevalence in *A. terrestris*. The influence of baiting was represented by the interaction between the two factors: area type (baited vs. nonbaited areas) and period (temporal progress of the experiment). The area type and period variables were added as blocking variables to the initial model. In addition, season (spring: March to June, summer/autumn: July to October, winter: November to February) and urban area variables (urban zone, border zone, and periurban zone) were included in the initial model since these factors were known to affect the prevalence of *E. multilocularis* (17).

Deviations from expected frequencies were tested by chi square tests. P values are given two-tailed if not otherwise stated. If the minimum entry in the table of expectation was <5, p values were calculated with Actus (George F. Estabrook, New Hampshire, USA), which performs randomized contingency tables and gives probabilities for deviations from expected values (29). Critical significance levels were Bonferroni-corrected according to Rice, taking into account multiple tests on the same data (30). We calculated exact binomial 95% confidence intervals (CI) for means of binomial variables, according to the method of Clopper and Pearson (31).

Results

Baiting and Environmental Contamination

To evaluate the effect of the experimental baiting, we analyzed 682 fox fecal samples collected in the six 1-km^2 bait areas and 523 fecal samples from the six control areas. The stepwise logistic regression indicated a significant final model (model χ^2=139.4, df=11, p<0.001) with a highly significant influence of anthelmintic baiting, expressed by the interaction between the area type and period variables on the proportion of coproantigen-positive fecal samples (Wald Statistics 20.5, df=4, p<0.001). The proportion of coproantigen-positive fecal samples in bait areas decreased from 38.6% (95% CI 26.0% to 52.4%) during winter 1999 to 5.5% (95% CI 3.1% to 8.9%) in summer/autumn 2001. In the control areas, the initial proportion of coproantigen-positive fecal samples was 47.1% (95% CI 35.1% to 59.4%) in winter 1999; it decreased to 25.4% (95% CI 15.3% to 37.9%) in the initial phase of baiting (spring 2000) but thereafter remained stable during the baiting experiment (Figure 2). The two blocking factors, period (Wald Statistics=60.9, df=4, p<0.001) (Figure 2) and urban area (Wald Statistics=6.0, df=2, p=0.05), also entered the final model. In the urban zone, 1 of 33 fecal samples was coproantigen positive (mean 3.0%; 95% CI 0.0% to 15.8%), whereas within the border zone and in the periurban zone, the proportion of coproantigen-positive fecal samples was significantly higher with similar percentages of 19.1% (95% CI 16.3% to 22.0%) and 18.5% (95% CI 14.8% to 22.6%).

A strong decrease in the proportion of coproantigen-positive fecal samples was also recorded in the 332 fecal samples collected in the 6–km^2 bait area. Before baiting started in winter 1999/2000, the proportion of coproanti-

gen-positive fecal samples was significantly higher than in the 1-km² bait areas (mean 66.7%; 95% CI 46.0% to 83.5%; χ^2 test: $p<0.05$). The proportion decreased significantly to 9.2% (95% CI 3.8% to 18.1%) during summer/autumn 2000, and to 1.8% (95% CI 0.0% to 6.5%) during summer/autumn 2001 (Actus randomization test, $p<0.001$). This proportion of coproantigen-positive fecal samples did not differ significantly from the final proportion of positive fecal samples found in the 1-km² bait areas.

The spatial persistence of the baiting effect was investigated by comparing the prevalence in relation to the distance from the baiting area. In both the bait area center (>250 m inside the bait area) and in the bait area periphery (250 m inside to the border of the bait area), the effect of baiting was very pronounced (Figure 3). For fecal samples collected 0 to 500 m from the next bait area, the effect of baiting was less clear; for those collected ≥500 m, no significant effect could be registered.

A total of 16 coproantigen-positive fecal samples from bait areas and 55 coproantigen-positive fecal samples from control areas, all collected from July to October 2001, were investigated for the presence of *E. multilocularis* eggs. PCR analyses revealed significantly fewer fecal samples positive for *E. multilocularis* eggs (mean 25.0%; 95% CI 7.3% to 52.4%) in bait areas than in control areas (mean 52.7%; 95% CI 38.8% to 66.3%; χ^2 test [one-tailed] $p<0.05$).

Prevalence in Intermediate Hosts

Of 1,014 *A. terrestris*, 509 originated from 1-km² bait areas and 505 from control areas. The stepwise backward logistic regression indicated a significant final model (model $\chi^2=8.4$, df=3, $p<0.05$) showing that anthelmintic baiting, expressed by the interaction between area type and period, on the prevalence of *E. multilocularis* in *A. terrestris* (Wald Statistics 3.7, df 1, p [1-tailed] <0.05) had an influence. During the first year of baiting, the prevalence in control and baited areas was similar (Figure 4), but during autumn 2001 the prevalence in baited areas was significantly lower (mean 2.1%; 95% CI 0.6% to 5.2%) than in control areas (mean 7.3%; 95% CI 4.4% to 11.2%). Independently from their interaction effect, the blocking variables of period and area type also entered the final model but not urban area and season.

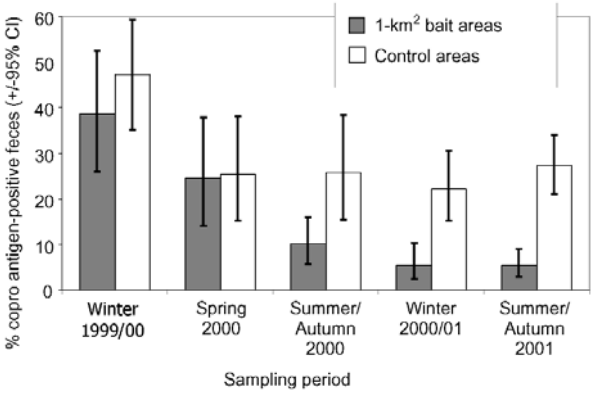

Figure 2. Proportions of *Echinococcus multilocularis* coproantigen–positive fox fecal samples and 95% exact binomial confidence intervals in the six 1-km² bait areas, baited monthly with 50 praziquantel-containing baits per km², and the six unbaited control areas during the experiment.

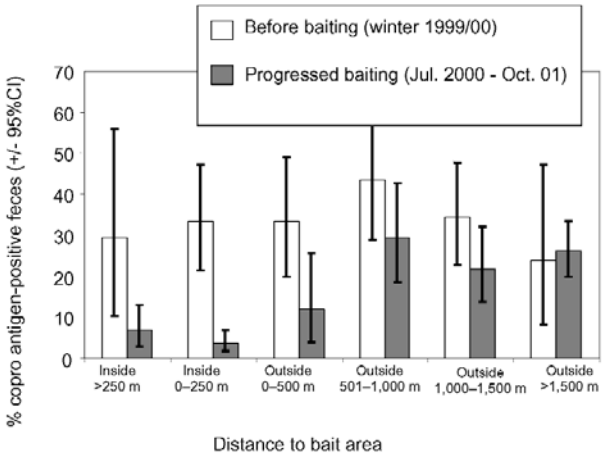

Figure 3. Proportions of *Echinococcus multilocularis* coproantigen–positive fox fecal samples and 95% exact binomial confidence intervals obtained at different distances from the border of the 1-km² bait areas, baited monthly with 50 praziquantel-containing baits per km², before baiting started (November 1999 to March 2000) and after baiting had taken place for 3 months (July 2000 to October 2001).

The results for the 1-km² bait areas could be confirmed in the 6-km² bait area. The prevalence of *E. multilocularis* in 215 *A. terrestris* was highest from July 1999 to February 2000 before baits were delivered (mean 21.6%; 95% CI 11.3% to 35.3%) and decreased significantly afterwards ($\chi^2=4.54$, df=2, p [1-tailed]=0.05). The prevalence was lower from April to November 2000 (mean 14.3%; 95% CI 6.4% to 26.2%) and lowest from July to October 2001 (mean 9.3%; 95% CI 4.5% to 16.4%).

Discussion

Baiting Strategy and Bait Density

The high bait density of 50 baits per km², combined with a manual bait distribution at sites attractive for foxes, was highly effective. In oral rabies vaccination campaigns, up to 20 baits were usually delivered per km² (32). Also, in the anthelmintic bait studies in Germany bait densities from 15 to 20 baits per km² were successfully used (19,20). In contrast to densities in rural habitats, in urban areas, fox densities can easily exceed 10 adult foxes per km² (23,24). Furthermore, in summer, many adolescent foxes are present. A previous camera trap study conducted

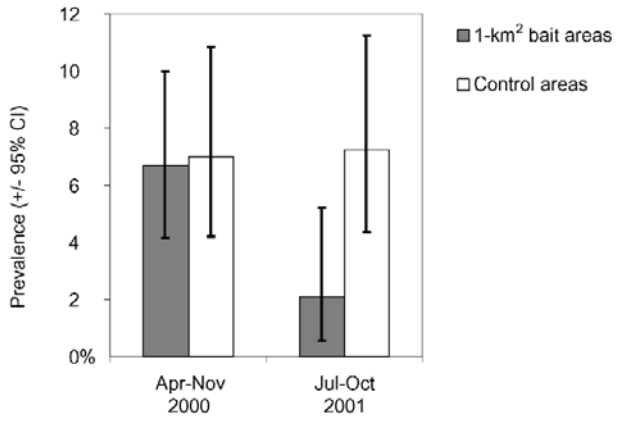

Figure 4. Prevalences of *Echinococcus multilocularis* in *Arvicola terrestris* and 95% exact binomial confidence intervals in the six 1-km² bait areas, baited monthly with 50 praziquantel-containing baits per km², and the six unbaited control areas during the experiment.

in Zürich showed that approximately half of the baits that disappeared were taken by foxes but the others were consumed by hedgehogs, dogs, rodents, and snails (Hegglin et al., unpub. data.). Therefore, bait densities exceeding 20 baits per km² seem to be appropriate to reach most foxes in urban habitats. In addition, the manual distribution of baits at selected sites attractive for foxes can improve bait uptake of foxes (Hegglin et al., unpub. data).

Small-Scale Anthelmintic Baiting

Our results show clearly that the *E. multilocularis* egg contamination in urban areas can be reduced to a low level by manually distributing anthelmintic baits at monthly intervals. This reduction is even possible within defined urban patches of 1-km² in areas where the organism is highly endemic. Although the initial rate of coproantigen-positive fecal samples was high (38.6%), this rate decreased to 5.4% during the first year of baiting. Additionally, the coproantigen-positive fecal samples in baited areas contained *E. multilocularis* eggs significantly less frequently. In contrast to our results, a large-scale praziquantel-baiting campaign in a rural area in southern Germany, covering 566 km², showed a strong effect in the 156-km² core area, but in the 6- to 10-km border area, the effect was much less pronounced (19). Immigration of young, infected foxes may have caused this border effect. In Hokkaido, Japan, an anthelmintic baiting study was carried out in a smaller, rural area of 90 km², which resulted in a drastic reduction of environmental contamination comparable to our study (21). The main difference between the German study, the Japanese study, and our own may be explained by the different baiting strategies. In Germany, approximately half of the baits were randomly delivered by aircraft, and the intervals between two baiting actions varied from 2 to 4 months. In our study, all baits were delivered manually around places attractive for foxes at monthly intervals. A model for *E. multilocularis* control indicated that baiting intervals of 4 to 6 weeks would be most efficient (33).

The strong local effect in this study shows that in urban areas the population dynamics of *E. multilocularis* is mainly determined by factors of very restricted spatial extension. Knowledge about spatial dynamic of fox populations is crucial in understanding the dispersion capacity of *E. multilocularis*. Urban settings, which provide plentiful food sources, are well-suited to sustain high population densities of foxes (34), who tend to have small home ranges and low dispersing distances (35,36). In addition, urban fox populations are generally organized in family groups, in which predominantly young vixens remain in the parental home range and help rear pups (36,37). Consequently, offspring frequently inherit parental territory and do not have to disperse. Furthermore, a low urban immigration rate, which has been substantiated by genetic microsatellite analyses for the Zürich urban fox population (38), and a low hunting pressure (see Methods) contribute to the moderate spatial dynamics of the urban fox population, which we assume to be a precondition for the effectiveness of the small-scale anthelmintic treatment.

Reduction of Infection Pressure

During the first year of baiting, when the proportion of *E. multilocularis*–coproantigen-positive fecal samples had already decreased significantly, no difference was detected in the *A. terrestris* prevalence of bait and control areas. The significantly lower prevalence of *A. terrestris* trapped in bait areas during the second year of baiting demonstrates that lower prevalence of *E. multilocularis* egg contamination resulted in a lower infection pressure for intermediate hosts. Nevertheless, at the end of the baiting study, *E. multilocularis* egg–containing fecal samples and infected intermediate host could still be detected in the 1-km² and the 6-km² bait areas. This finding shows that the life cycle of the parasite in the baited areas was not completely interrupted. Dispersing and transient foxes can always contaminate baited areas, even in much larger areas. Furthermore, eggs of this cestode are stable under suitable environmental conditions (39), infected intermediate hosts can stay infectious over several months (40), and baited foxes can become reinfected just after treatment by consuming an infected intermediate host. In addition, the intervention studies in Germany (19,20) demonstrated that *E. multilocularis* has the potential to recover from a population breakdown in >2 years (T. Romig, pers. comm.; K. Tackmann, pers. comm). Therefore, a baiting strategy that focuses on extinction of the parasite in large areas might fail, and permanent intervention to lower *E. multilocularis* egg con-

tamination in defined risk areas might be more realistic and cost efficient.

Conclusion

We demonstrated the feasibility of small-scale anthelmintic baiting of foxes to reduce *E. multilocularis* egg contamination in urban areas intensively used by the public for recreational activities, such as gardening or outdoor sports. In addition, the lower prevalence of infected voles also reduces the risk of domestic carnivores becoming infected by preying on voles and, consequently, the risk for egg transmission to pet animals. Therefore, we recommend that public health policy should focus on such defined areas where the organism is highly endemic to reduce a potential risk for alveolar echinococcosis.

Acknowledgments

The authors thank Claudia Stieger, Isabelle Tanner, Rita Bosshart, Gioia Schwarzenbach, Sasa Stefanic, and all other collaborators for help in the extensive field and laboratory work; Christian Stauffer and the game wardens of the city of Zürich for their generous collaboration; and Fabio Bontadina and Paul Torgerson for valuable comments on an earlier draft of the manuscript.

This investigation was carried out within the context of the Integrated Fox Project, an interdisciplinary research project on the dynamics of the fox population in Switzerland. The study was supported by the Swiss Federal Office of Veterinary Medicine, Berne (Projekt Nr. 1.99.03) and the Swiss Federal Office for Education and Science (EchinoRisk Projekt QLK2-CT-2001-001995 / BBW Nr. 00.0586-2).

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Cephalosporin-resistant *Escherichia coli* among Summer Camp Attendees with Salmonellosis

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Investigation of an acute gastroenteritis outbreak involving >100 persons at a summer camp in Girona, Spain, in June 2002 led to the detection of *Salmonella enterica* and extended-spectrum cephalosporin-resistant *Escherichia coli* (ESCREC). Stool cultures were performed for 22 symptomatic campers, three asymptomatic food handlers, and 10 healthy household members. Of the 22 campers, 19 had *Salmonella enterica*, 9 had an ESCREC strain carrying an extended-spectrum β-lactamase, and 2 had a second ESCREC strain carrying a plasmidic cephamycinase. Related ESCREC were detected in two (salmonella-negative) asymptomatic food handlers and in none of the healthy household members. Fecal ESCREC and its β-lactamases and plasmids were extensively characterized. Three of the five ESCREC clones were recovered from multiple hosts. The apparent dissemination of ESCREC suggests a food or water vehicle. The observed distribution of resistance plasmids and β-lactamase genes in several clones indicates a high degree of horizontal transfer. Heightened vigilance and increased efforts must be made to discover the reservoirs and vehicles for community dissemination of ESCREC.

Strains of *Escherichia coli* that produce enzymes capable of degrading extended- spectrum cephalosporins (ESCs), i.e., extended-spectrum β-lactamases (ESBLs), or these drugs plus cephamycins, i.e., plasmidic or hyperproduction of chromosomal cephamycinases have recently emerged as important nosocomial pathogens (1,2). Some of these strains cannot be reliably detected by clinical microbiology laboratories by using conventional susceptibility tests (3), and even when recognized, treating infections caused by these strains can be challenging because therapeutic options are limited. Infections attributable to such strains are associated with prolonged hospital stays, increased healthcare costs, and an increased number of deaths if appropriate therapy is delayed (4,5).

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To date, almost all reports of infection or colonization with ESBL- and plasmidic cephamycinase-producing *E. coli* (i.e., extended-spectrum cephalosporin-resistant *E. coli* [ESCREC]) have involved hospitalized patients or nursing home residents (3,6). The few reported patients with community-acquired infection have been elderly and debilitated and have had hospital contact, important coexisting conditions, or both (3,6).

E. coli, including resistant strains, may be transmitted within the community through the food supply. Indeed, other gram-negative enteric pathogens, notably *Salmonella enterica*, are a frequent cause of foodborne disease and, increasingly, are associated with antibiotic resistance, including antibiotic resistance to ESCs (7–11). Available data regarding other resistant *E. coli* suggest that ESCREC could also be disseminated through the food supply (12–19).

The cefoperazone-containing medium routinely used in our laboratory for the isolation of *Campylobacter* occasionally yields other bacteria with hyperproduction of chromosomal β-lactamases or their plasmidic derivatives, as well as strains carrying extended-spectrum β-lactamases (unpub. data). By using this media, we have isolated several resistant enterobacteriaceae strains from patients with sporadic cases of gastroenteritis (unpub. data). During an investigation of a summer camp-associated salmonellosis outbreak, we observed that stool cultures from nine campers unexpectedly yielded, on cefoperazone-containing medium, colonies resembling enterobacteriaceae, with a uniform mucoid appearance. This result suggested the possibility that the same, probably ESC-resistant, enterobacterial strain was present in all these persons, findings consistent with possible foodborne spread. Consequently, all samples were reevaluated on media containing cefotaxime (see Methods) to increase sensitivity for detection of ESC-resistant organisms. To gain more knowledge of foodborne spread as a potential mechanism of dissemination of resistance genes, we undertook an extensive molecular epidemiologic analysis of these isolates.

Methods

Description of the Outbreak and Stool Sampling

Two hundred twenty-five elementary and secondary school students and 11 teachers were spending a week (June 11–15, 2001) at a summer camp in Palafrugell, Girona (Spain), when an outbreak of gastroenteritis began on June 14. An epidemiologic investigation involving 200 campers and staff failed to identify the source of the outbreak. Clinical and epidemiologic studies were initiated at the onset of the outbreak, but our participation as reference laboratory began later. Consequently, a limited number of stools from symptomatic patients and related persons were available to us for analysis.

From June 16 to 19, stool samples from 22 ill campers were collected for analysis. On July 6 (18–19 days later), additional stool samples were collected from four ill campers and from 10 asymptomatic household members of the four ill campers. Stool samples were also collected from three asymptomatic food handlers, on June 19, 22, and 28.

Microbiologic Studies

Human Samples

Stool samples were processed according to conventional protocols for isolation of enteropathogenic bacteria, in addition to special selection for resistant organisms (as described below). Isolates were identified by conventional methods (20). Stools (fresh and after modified Ziehl-Neelsen staining) were microscopically examined for protozoa (21). Latex agglutination was used to detect rotavirus immunochromatography to dectect adenovirus 40/41, and reverse transcriptase–polymerase chain reaction (RT-PCR) (22) to detect calicivirus.

Selection of ESCREC

Stool suspended in saline was added to trypticase soy broth containing 2 mg/L cefotaxime (TSB-CTX), which after 18 h of incubation at 35°C, was added to similarly supplemented MacConkey broth (MacC-broth-CTX). After an 18-h incubation, a loopful of this broth was spread on similarly supplemented MacConkey agar. A colony of each distinct morphotype was analyzed further.

Selection of Resistant *S. enterica* Strains

Stool samples were processed as for isolation of ESCREC, but after the initial growth the MacC-broth-CTX was added to selenite broth. After 8 h of incubation at 35°C, this broth was spread on cefotaxime-supplemented xylose-lysine-deoxycholate agar. Colonies suspected of representing *Salmonella* were analyzed further.

Food Studies

Eight unprepared food items from the camp kitchen were analyzed for *Salmonella* and ESCREC as described for stool samples, except that the initial TSB-CTX was replaced by peptone yeast extract broth.

Antibiotic Susceptibility Testing

Disk diffusion susceptibility testing to 28 antibiotics (Table) was performed according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines (23). The activity of cefotaxime and ceftazidime, combined with clavulanate, was determined by E test (AB Biodisk, Solna, Sweden). MICs to β-lactam antibiotics were determined by broth microdilution (Sensititre, Trek Diagnostic Systems LTD, West Sussex, England), according to NCCLS guidelines (24,25).

Transfer of Resistance Determinants

Filter matings were performed with ESCREC donors, using as recipients either *E. coli* HB101 (Nal Kan) for which transconjugants were selected, according to described methods (26), or the *S. enterica* isolate from patient P12, for which transconjugants were selected by adding the filter containing mixed growth of *E. coli* and *Salmonella* to MacC-broth-CTX. This broth, which was subsequently processed as described above for selection of resistant *S. enterica*.

Extraction of β-Lactamases and Isoelectric Focusing (IEF)

Crude extracts of β-lactamases were obtained by ultrasonication. Analytical IEF was performed as previously described (27).

Characterization of β-Lactamase Genes

The bla_{TEM}, $bla_{\text{CTX-M-9}}$ and $bla_{\text{CMY-2}}$ genes were amplified as previously described (28–33). The DNA sequence was directly determined from PCR products for both DNA strands (34).

Biotyping and Serotyping

The biotype, as determined for 12 metabolic reactions, was expressed as a 4-digit code (35). The serotype and phage type of *Salmonella* isolates were determined in the Servicio de Enterobacterias del Centro Nacional de Microbiología, Instituto Carlos III, Majadahonda, Spain (M.A. Usera and A. Echeita). The serogroup of the ESCREC was determined at the Laboratorio de Referencia, Lugo, Universidad de Santiago de Compostela (J. Blanco).

Pulsed-Field Gel Electrophoresis (PFGE)

Genomic profiles were analyzed by PFGE with *Xba*I (Amersham Biosciences UK Limited, Buckinghamshire,

Table. Characteristics of 15 extended-spectrum cephalosporin-resistant fecal *Escherichia coli* isolates derived from an outbreak of salmonellosis

Patient no. (age in y)	School[b]	Isolate	Isolation date	Biotype	Serotype	PFGE pattern (clone)	Plasmid profile	Southern blot pattern		β-lactamase	Associated resistance[d]
								*Pst*I	*Sma*I / *Hinc*II[c]		
P5[a] (12)	EP	1	06-18-01	5671	NT	A	I a[e]	1	a	CTX-M-9	Tp, Sxt
P6[a] (11)	EP	1	06-18-01	5671	NT	A	I b[e]	2	b	CTX-M-9	Tp, Sxt
P9[a] (12)	SB	1	06-19-01	5671	NT	A	I c[e]	2	b	CTX-M-9	Tp, Sxt
P10[a] (12)	SB	1	06-19-01	5671	NT	A	I a[e]	3	c	CTX-M-9	Tp, Sxt
		2	06-19-01	7775	O:86	B	II a[e]	4	d	CMY-2	Tp, Sxt, Cm, Fur
P12[a] (11)	SB	1	06-19-01	5671	NT	A	I b[e]	3	b	CTX-M-9	Tp, Sxt
		2	07-06-01	5671	NT	A	I b[e]	3	b	CTX-M-9	Tp, Sxt
P14[a] (12)	SB	1	06-19-01	1571	O:20	C	III	3	e	CTX-M-9 + TEM-1	Tp, Sxt, Km, Neo
P15 (11)	SB	1	06-19-01	0371	O:145	D	IV	3	f	CTX-M-9 + TEM-1	Tp, Sxt, Cm
P18[a] (11)	SB	1	06-19-01	5671	NT	A	I b[e]	3	b	CTX-M-9	Tp, Sxt
		2	06-19-01	7775	O:86	B	II b[e]	4	d	CMY-2	Cm, Fur
P19[a] (12)	T	1	06-19-01	5671	NT	A	I b[e]	3	b	CTX-M-9	Tp, Sxt
F2		1	06-22-01	1571	O:20	C	V	4	nd[f]	CTX-M-9 + TEM-1	Tp, Sxt, Km, Neo
F3		1	06-22-01	4571	O:55	E	VI	4	d	CMY-2 + TEM-1	Cm, Gm, Km, Neo, Tob
		2	06-28-01	4571	O:55	E	VI	4	d	CMY-2 + TEM-1	Cm, Gm, Km, Neo, Tob

[a]*Salmonella enterica* also was isolated.
[b]SB, Sant Boi; T, Tarragona; EP, El Prat.
[c]The *Sma*I RFLP pattern was determined for those strains carrying the CTX-M-9 enzyme and the *Hinc*II RFLP pattern was determined for those carrying the CMY-2 enzyme.
[d]All ESCREC isolates were resistant to all penicillins and cephalosporins, including ESCs, as well as to nalidixic acid, tetracycline, sulfonamides and streptomycin. Other antibiotics tested included ampicillin, amoxicillin-clavulanate, piperacillin, cefazolin, cefuroxime, cefoxitin, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, chloramphenicol (Cm), gentamicin (Gm), kanamycin (Km), tobramycin (Tob), amikacin, neomycin (Neo), sulfamethoxazole-trimethoprim (Sxt), trimethoprim (Tp), ciprofloxacin, fosfomycin, nitrofurantoin, rifampin and furazolidone (Fur).
[e]The only difference between the Ia, Ib, and Ic, or the IIa and IIb plasmid profiles is one band higher than 100 kb within the particular plasmid pattern.
[f]nd, not determined.

England) (36,37). Isolates exhibiting indistinguishable PFGE profiles were considered to represent the same clone.

Plasmid Profiles Analysis

Plasmid DNA was isolated by using a commercial kit (QIAGEN, Inc., Valencia, CA) and subjected to 0.8% agarose gel electrophoresis both without digestions and after cleavage with *Pst*I, *Sma*I, or *Hinc*II (Amersham Biosciences). For southern hybridization (38), both total and restricted plasmid DNA were transferred to nylon membranes and hybridized with PCR-generated probes for $bla_{CTX\text{-}M\text{-}9}$ (850 bp) and $bla_{CMY\text{-}2}$ (1,017 bp), as labeled and detected using the ECL Direct Nucleic Acid Labeling and Detection System (Amersham Biosciences).

Statistical Methods

Comparisons of proportions were tested by using the Fisher exact test (two-tailed).

Results

Epidemiologic Survey

The 225 student campers, 10–16 years of age, and 11 teachers were from three schools in three cities, Tarragona (T), El Prat (EP), and Sant Boi (SB). Of the 200 campers and staff interviewed, 109 (54.5%), including 3 teachers, had symptoms of gastroenteritis, with no significant differences between the three schools (57%, 49%, and 62% respectively). The most frequent symptoms were abdominal pain (80%), diarrhea (79%), and headache (64%). Two students were admitted to hospital, but after supportive therapy were discharged within 48 hours. No person received antibiotic therapy.

Microbiologic Study

Campers

Of the 109 ill campers, 22 provided stool samples that were available for microbiologic study in our laboratory. Nineteen (86%) of the acute-phase stool samples yielded

S. enterica serovar Enteritidis phage type 4, which was susceptible to all tested antimicrobial agents. No fecal pathogens were detected in the remaining three ill campers.

In addition, ESCREC were isolated from the initial stool sample for 9 (41%) of the 22 campers, including 8 (42%) of 19 with salmonellae and 1 with no detectable enteric pathogen (Table). All nine samples contained an ESCREC isolate resistant to penicillins and cephalosporins (including ESCs) but susceptible to cephamycins and carbapenems (CTX^R-FOX^R-ESCREC), consistent with production of an ESBL. Two of these samples also yielded a second ESCREC type that was resistant to penicillins and cephalosporins, including ESCs and cephamycins, but susceptible to carbapenems (CTX^R-FOX^R-ESCREC), consistent with hyperproduction of *E. coli* AmpC chromosomal β-lactamase or presence of a plasmidic cephamycinase. All ESCREC isolates exhibited multiple additional resistance markers (Table).

Follow-up stool samples (collected 18–19 days later) were available for four campers. Neither salmonellae nor ESCREC was recovered, except in one camper, from whom the initial ESCREC strain was isolated (Table).

Food Handlers and Households Contacts

Although stool samples collected from the three (asymptomatic) food handlers were negative for enteropathogens, two of these persons were carriers of ESCREC. One had CTX^R-FOX^S-ESCREC at the second sampling, whereas the other had CTX^R-FOX^R-ESCREC at both the second and third samplings.

In contrast, neither enteropathogens nor ESCREC were detected in stool samples from 10 healthy household members of four ill campers who had both ESCREC and salmonellae in their acute-phase stool sample (for prevalence of ESCREC among household members vs. campers or food handlers, p=0.03 and p=0.04, respectively).

The eight cultured camp food items yielded neither *S. enterica* serovar Enteritidis nor ESCREC.

E. coli β-Lactamases

In the 11 *E. coli* isolates phenotypically suspected of ESBL production, a β-lactamase with an isoelectric point of 8.0 was detected. PCR with $bla_{CTX\text{-}M\text{-}9}$-specific primers and sequence analysis confirmed the presence of the CTX-M-9 β-lactamase (not shown).

Three of these isolates had an additional β-lactamase with pI 5.4, which in IEF reacted with penicillin but not with ceftriaxone. PCR with bla_{TEM}-specific primers confirmed the presence of a TEM-1-like β-lactamase (Table).

All four isolates phenotypically suspected of AmpC β-lactamase production were PCR-positive with *ampC*-family primers. Sequence analysis confirmed the presence of the CMY-2 β-lactamase (not shown).

Biotyping, Serotyping, and PFGE Analysis of Isolates

Collectively, the 15 ESCREC isolates represented five distinct biotypes (Table). Overall, five serogroups (O20, O55, O86, O145, and non-typable/NT) and five PFGE pulsotypes (A to E) were detected. Biotype, serogroup, and pulsotype corresponded precisely, confirming the presence of five discrete ESCREC clones (Table and Figure 1).

Epidemiologic Distribution of Clones

Clone A (CTX-M-9), the most prevalent, was recovered from students from all three cities, EP, SB, and T (Table). Clone B (CMY-2) was recovered from two students from Sant Boi and clone E (also CMY-2) was recovered from a food handler. Clone C (CTX-M-9 + TEM-1) was recovered from both a Sant Boian student and a food handler. Clone D (CTX-M-9 + TEM-1) was recovered from a Sant Boian student.

Plasmid Analysis

Plasmid profiles of the ESCREC isolates were largely concordant with clonal assignments (Figure 2A, Table). In all but one isolate, the probes for $bla_{CTX\text{-}M\text{-}9}$ and $bla_{CMY\text{-}2}$ hybridized to a single large (>150 kb) plasmid (Figure 2).

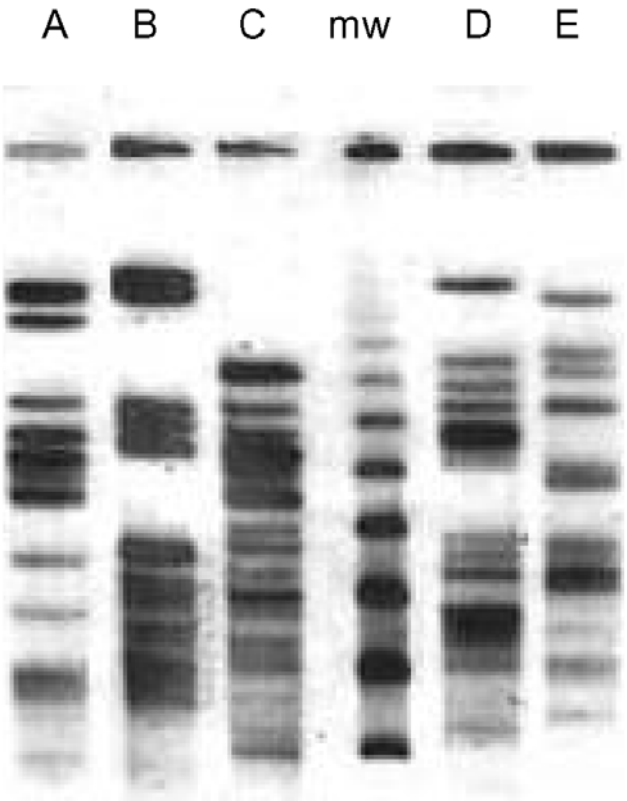

Figure 1. Restriction pattern (*Xba*I) by pulsed-field gel electrophoresis of the five extended-spectrum, cephalosporin-resistant *Escherichia coli* clones (A, B, C, D, E). mw: marker.

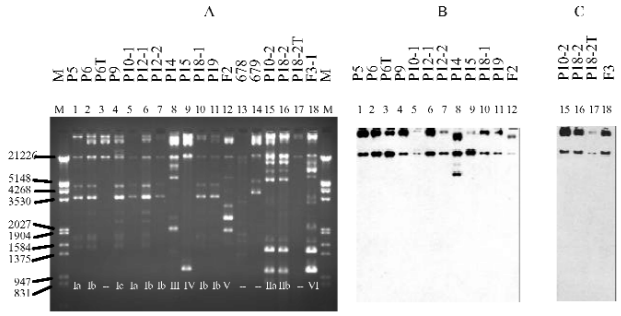

Figure 2. Plasmid profile (A) and hybridization with CTX-M-9 probe (B) and CMY-2 probe (C). The studied isolates, by lane, are: 1: P5, 2: P6, 3: P6T (transconjugant of P6), 4: P9, 5: P10-1, 6: P12-1, 7: P12-2, 8: P14, 9: P15, 10: P18-1, 11: P19, 12: F2, 15: P10-2, 16: P18-2, 17: P18-2T (transconjugant of P18-2), 18: F3-1, 13, and 14: plasmid control strains *E. coli* 678 CECT (= NCTC 50193 with the following plasmid sizes: 54.38, 7.30, 5.56, 5.14, 3.98, 3.08, 2.71, and 2.06 kb) and *E. coli* 679 CECT (= NCTC 50192 with the following plasmid sizes: 148.5, 63.8, 36.2 and 7 kb.). M is marker III (Roche Diagnostics GmbH, Mannheim, Germany). Below each lane in panel A is indicated the plasmid profile designation shown in the Table.

By Southern blot analysis, the $bla_{CTX\text{-}M\text{-}9}$-containing plasmids (clones A, C, and D) exhibited minor diversity within and greater diversity among clones (Figures 2–4, Table). In the uncut plasmid blot, the large, probe-positive plasmids of clones A and D were uniform in size and larger than those of clone C (Figure 2B). The corresponding *Pst*I Southern blot showed three different patterns, which were not clone-specific (Figure 3, Table). All of these isolates exhibited a band at ~1,289 bp (Figure 3B), whereas three isolates (all clone A) also exhibited a variably sized larger band (Figure 3B, Table). The corresponding *Sma*I blot showed five patterns among the CTX-M-9-positive isolates, including three closely related patterns among the clone A isolates and unique patterns each for the clone C and D isolates (Figure 4, Table).

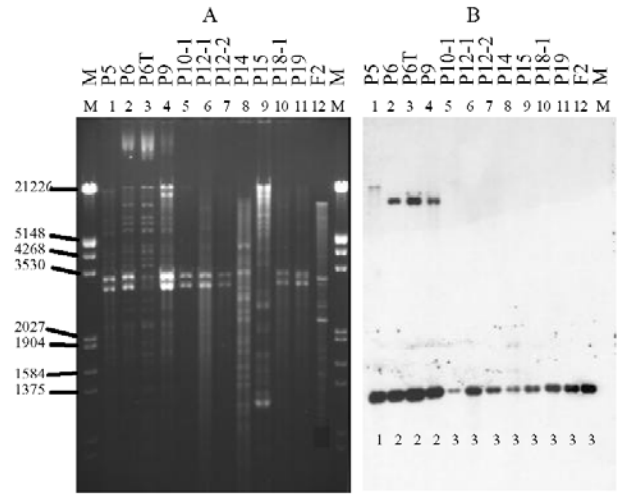

Figure 3. Plasmid restriction with *Pst*I (A) and hybridization of isolates carrying the CTX-M-9 enzyme with the CTX-M-9 probe (B). The isolates are as listed in Figure 1. Below each line of hybridization, the pattern shown in the Table is indicated.

In contrast, the $bla_{CMY\text{-}2}$-containing isolates (clones B and E) were indistinguishable in both the uncut plasmid blot (Figure 2C) and the *Pst*I and *Hinc*II blots, which showed a homogeneous single-band pattern for all three isolates (not shown).

Transfer of Resistance in vitro

One CTX-M-9-positive isolate transferred its resistance by in vitro conjugation to *E. coli* HB101 but not to a *S. enterica* isolate from a patient. In contrast, a CMY-2-positive isolate was successfully conjugated with this *S. enterica* isolate but not with *E. coli* HB101 (probably due to the donor strain's production of a bacteriocin that inhibits HB101; data not shown).

Discussion

Our microbiologic evaluation of an outbreak of *Salmonella* gastroenteritis at a summer camp uncovered the unsuspected dissemination among campers and camp staff of multiple clones of ESCREC containing diverse conjugally transferable β-lactamases. Several lines of evidence indicated that dissemination of ESCREC occurred within the summer camp. Sharing of ESCREC clones was observed among multiple hosts who had no contact with one another before camp, yet at camp lived together and shared a common food and water supply. Isolation of ESCREC was limited to camp attendees, to the exclusion of members of the campers' households who did not attend camp. Finally, the high observed prevalence of fecal ESCREC among camp attendees (11/25, 44%) contrasts strikingly with the low prevalence of ESCREC detected by using similar methods in reference fecal samples from 707 outpatients without an infectious disease diagnosis who attended Sant Pau Hospital from February through May, 2001 (2%: $p<0.001$; [39]) and among *E. coli* isolates from our hospital clinical microbiology laboratory (e.g., in 2000, for CTX-M-9, 0.5%; for CMY-2, 0.2%) (29).

The mechanism for dissemination of ESCREC within the camp remains undefined. Direct person-to-person spread is possible but seems unlikely, since transmission to household members after campers returned home was not evident, as would be expected if domestic contact could lead to transmission. However, hygienic conditions conceivably were worse at the camp, particularly during the outbreak of gastroenteritis.

The concurrent outbreak of salmonellosis, a classic foodborne pathogen, suggested that contaminated food (or possibly water) might have served as a vehicle for ESCREC within the camp. Indeed, ESCREC clones A and B were confined to hosts who also had salmonellae. Since

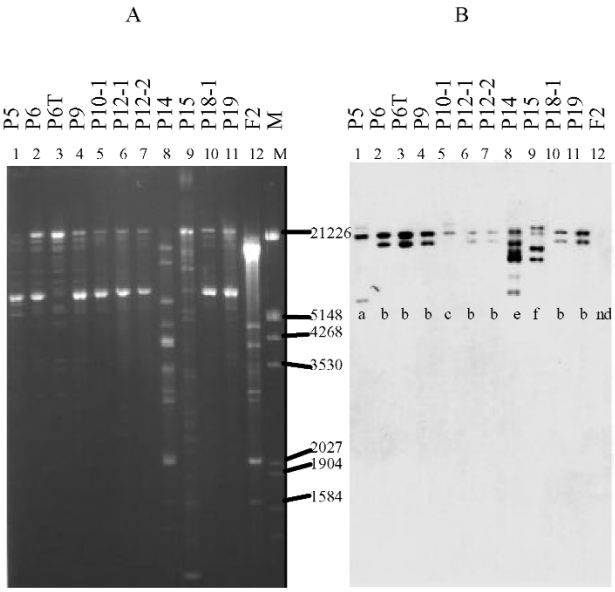

Figure 4. Plasmid restriction with *Sma*I (A) and hybridization of isolates carrying the CTX-M-9 enzyme with the CTX-M-9 probe (B). The isolates are as listed in Figure 1. Below each line of hybridization, the pattern shown in the Table is indicated.

only eight food items were cultured, the failure to recover ESCREC from camp foods provides little evidence to rule out foodborne transmission.

Although food handlers have been implicated in many foodborne outbreaks of intestinal disease (40), in this instance they appeared an unlikely source for either ESCREC or salmonellae. None of the three food handlers was colonized with salmonellae or with ESCREC clones A, B, or D, all of which were present in one or more campers, whereas one food handler had a unique ESCREC clone (clone E), one shared a distinct ESCREC clone (clone C) with a single camper, and one had no detectable ESCREC. Thus, if food were the vehicle, the contamination most likely occurred before the food's arrival at the camp, i.e., during production, processing, or transport. Since CMY-2 is closely associated with food animals, the present CMY-2-positive ESCREC plausibly could be of food animal origin. In contrast, CTX-M-9 and other ESBLs have been described only in humans. Thus, their presence suggests a human source of contamination.

Plasmid analysis indicated that although distinctive CTX-M-9-encoding plasmids were present in clones A, C, and D, the constituent $bla_{CTX\text{-}M\text{-}9}$ genes clearly derived from a common source, as demonstrated by their internal sequence identity and conserved flanking *Pst*I sites, despite the diversity of flanking *Sma*I sites. Minor within-clone diversity was evident among the $bla_{CTX\text{-}M\text{-}9}$-containing plasmids of clones A and C, consistent with recent microevolution. In contrast to the CTX-M-9-positive clones, clones B and E appeared to share the same $bla_{CMY\text{-}2}$-containing plasmid, consistent with recent horizontal transfer. Since clone B was isolated from two different hosts, it presumably acquired the CMY-2 plasmid before dissemination. By contrast, since clone E was recovered from only one host, the timing of its acquisition of the plasmid could not be determined. The diffusion of indistinguishable plasmids between different clones and the presence of similar but distinct plasmids within the same clone indicate the rapid biological dynamics of plasmids (9,10).

Conjugal transfer in vitro of broad-spectrum β-lactamases was achieved from ESCREC isolates to both a laboratory strain of *E. coli* (CTX-M-9) and an outbreak *S. enterica* isolate (CMY-2). These findings, which are consistent with our previous work and that of others (30,31), suggest that intraspecies or intergeneric transfer of broad-spectrum β-lactamases could occur in nature, either in vivo (in humans or animals) or in an inanimate reservoir (e.g., sewage or manure) (9,10). Thus, ESCREC may pose a threat both because of their direct potential for causing drug-resistant infections and because they can serve as vector of resistance elements for transmission to other pathogens or opportunistic microorganisms.

Our findings provide novel evidence of the dissemination of ESCREC among otherwise healthy persons. Moreover, in two persons ESCREC strains were documented to persist for at least 6 days or 17 days, suggesting possible establishment of stable colonization. Although no drug-resistant *E. coli* infections were known to have resulted from this dissemination, the data nonetheless suggest the possibility of widespread future emergence within the community of *E. coli* that is resistant to ESCs and of the responsible resistance genes, which could have substantial adverse health consequences (4,5).

Limitations of this study include the modest sample size (particularly for household members), short longitudinal follow-up, unusual circumstances (concurrent salmonellosis outbreak, summer camp setting), and absence of data regarding prior antibiotic use and hospital contact. Future studies should seek ESCREC in a similar setting, and in the general community, during a period without a known infectious disease outbreak.

In summary, our microbiologic evaluation of an outbreak of *Salmonella* gastroenteritis at a summer camp showed the unsuspected dissemination among campers and staff of multiple clones of ESCREC that contained diverse, conjugally transferable β-lactamases. Dissemination of ESCREC within the summer camp, possibly through food or water, was suggested by several lines of evidence. Confirmation of community-based transmission of ESCREC in other contexts and locales would indicate a need for heightened vigilance and efforts to discover the

reservoirs and vehicles for dissemination of ESCREC within the community.

Acknowledgments

We thank Jorge Blanco for the serotyping of *Escherichia coli* strains; Pilar Cortés, Montse Sabaté, and Laura Gómez for technical support; and Irene Barrabeig, Sofia Minguell, and Rosa Sala for collecting samples and clinical data from patients.

This study was partially supported by grants 97/0623 98/1293 from the "Fondo de Investigaciones Sanitarias de la Seguridad Social de España" (G.P.) and National Research Initiative Competitive Grants Program/ United States Department of Agriculture grant 00-35212-9408 (J.R.J.).

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Multijurisdictional Approach to Biosurveillance, Kansas City

Mark A. Hoffman,* Tiffany H. Wilkinson,† Aaron Bush,* Wayne Myers,* Ron G. Griffin,† Gerald L. Hoff,† and Rex Archer†

An electronic reporting system for a network of 22 laboratories was implemented in Kansas City, Missouri, with an independent organization acting as a data clearinghouse between the reporting laboratories and public health departments. The system ran in tandem with conventional reporting methods. Laboratory test orders and results were aggregated and mapped to a common nomenclature. Reports were delivered through a secure Internet connection to the Kansas City Health Department (KCHD); during the first 200 days of operation, 359 qualified results were delivered electronically to KCHD. Data were received more quickly than they were with conventional reporting methods: notification of chlamydia cases arrived 2 days earlier, invasive group A streptococcal disease cases arrived 2.3 days sooner, and salmonellosis cases arrived 2.7 days sooner. Data were more complete for all demographic fields, including address, age, sex, race, and date of birth. Two hundred fourteen cases reported electronically were not received by conventional means.

Biosurveillance is the automated monitoring of information sources of potential value in detecting an emerging epidemic, whether naturally occurring or the result of bioterrorism. Information sources that can be monitored for early warning include purchases of nonprescription medication (1) and symptoms reported during ambulatory care (2). Although these sources offer opportunities for early detection, they may also lead to high rates of false-positive reactions. A more definitive tool for biosurveillance is the electronic reporting of diagnostic results confirming the presence of a pathogen.

Heightened concerns about bioterrorism have led public health organizations to reevaluate methods used to report diseases. Currently, most healthcare providers notify public health organizations of reportable diseases by telephone, fax, or mail (3). These techniques generally delay the communication of confirmatory test results and notification of the appropriate public health organization (4). Underreporting is a major concern with traditional disease surveillance strategies (5); even cases of severe diseases sometimes go unreported (6). In addition, substantial variability exists in the completeness of the information sent to public health; initial reports often include only the test result and the patient name. They lack demographic details that are useful to public health officials, requiring them to perform followup calls to get the additional information (7). These delays and inconsistencies may impair the ability of public health officials to detect or respond to a bioterrorist event. One solution to these deficiencies is to use an electronic system to report disease to public health authorities.

Three approaches to electronic disease reporting are feasible. The first approach (Figure 1A) requires each healthcare provider to standardize clinical results (i.e., by using the Systematized Nomenclature of Medicine [SNOMED]) before sending results electronically to the appropriate authority. Researchers in Pittsburgh, working in an integrated delivery network that used a single data dictionary to minimize the difficulty of reconciling disparate coding systems, found this approach effective for electronic disease reporting (8). However, developing this type of system can be challenging because of the difficulty in updating multiple data dictionaries (translation tables that associate terms from multiple organizations). The second approach (Figure 1B) requires the use of result standardization software (which collects data from multiple sites and attempts to automatically associate terms to standard terms) at public health facilities. This approach places the responsibility for technology and personnel with the public health organizations. The third approach to electronic reporting (Figure 1C) involves an intermediary organization that aggregates the data and distributes standardized reports to public health organizations. Single jurisdiction systems using this approach have been developed in Hawaii and Indianapolis (9,10). We developed a multiple-jurisdiction data clearinghouse system (11) and assessed the benefits of the system in terms of timeliness, data completeness, and geographic depth of coverage.

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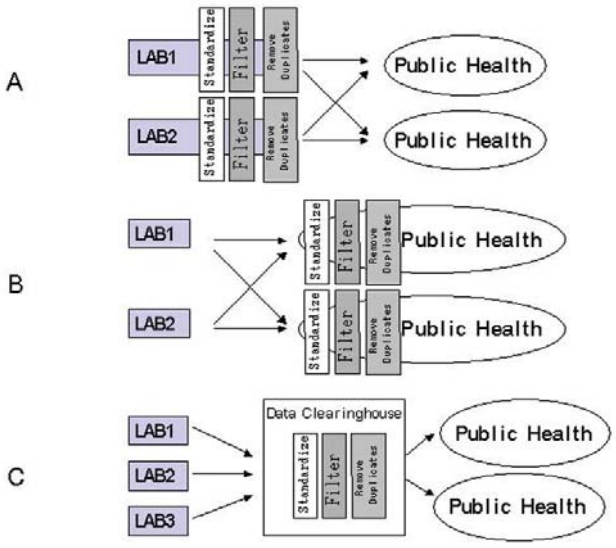

Figure 1. Comparison of technical approaches to biosurveillance: A) standardization, filtering, and checking for duplication done at contributor site; B) translation and checking for duplication at public health site; C) data repository.

Methods

The electronic reporting system consisted of three participating groups: data contributors, the clearinghouse, and public health organizations (Figure 2). Four nonaffiliated healthcare organizations of varying size (consisting of 2, 2, 5, and 13 facilities) participated in the system. The smallest facilities were regional care centers with 49 beds each; the largest was an urban hospital with 650 beds. All participating organizations used the same laboratory information system (LIS) (PathNet, Cerner Corp., Kansas City, MO) to document clinical microbiology results. Microbiology reports were constructed from a combination of codified entries representing the pathogen and discrete observations or free text entries added by the user. The data dictionaries and test catalogs at each organization were unique and were not referenced to standardized vocabularies. During the implementation of the electronic reporting system, the data dictionaries and test catalogs from the participating organizations were uploaded to the data clearinghouse; entries representing reportable pathogens or tests with the potential to yield reportable results were mapped to a standardized vocabulary.

We established agreements to assure security within the system. Surveillance data reported to public health organizations are exempted from the Health Insurance Portability and Accountability Act. Legal agreements designating the supplier of the clearinghouse system as a business associate with full reporting capability were established with the participating organizations. These agreements, combined with stringent access controls (physical and procedural security measures), encryption of identifiable information, and virtual private network (VPN) secured communications and ensured protection of confidentiality. Interagency agreements between public health organizations in the Kansas City area provided a further framework for the appropriate exchange of data across jurisdictional boundaries. For example, an agreement between KCHD and the state of Kansas recognized Kansas City, Missouri, as a county of Kansas for purposes of metropolitan surveillance.

Batch extraction scripts ran daily at each data contributor, pulling new laboratory test orders, laboratory results, and patient demographic information. Demographic information was encrypted before being transferred to the data clearinghouse through a VPN (Cisco Systems, San Jose, CA). The data were loaded into a data warehouse (Oracle Corp., Redwood Shores, CA) and checked for errors and duplication; data mapping was then performed (Informatica Corp., Redwood City, CA). Microbiologic results identifying reportable pathogens were mapped automatically to a common nomenclature to standardize the varying names between the participating organizations. Procedure orders (i.e., stool culture) deemed relevant to the detection of an infectious disease outbreak were also mapped to a common nomenclature. After these processing steps, the results were used to build two reports that were delivered through the Internet to KCHD using a VPN secured account. One report provided trending information on orderable procedures; the other provided results from microbiology tests (Figure 3). KCHD staff reviewed the reports using a report viewer (BusinessObjects Corp., San Jose, CA). Reports summarizing the results reported to KCHD were also delivered to the institution of origin.

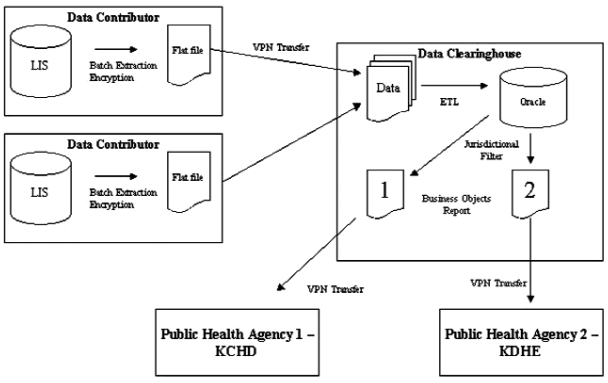

Figure 2. Data clearinghouse system architecture. Data are extracted from the laboratory information network at contributing sites and encrypted into a flat file. These are then delivered by virtual private network (VPN)–secured file transfer protocol to the clearinghouse where they are subjected to data warehousing processes. Jurisdictional filters are applied to the data to construct reports with data appropriate for the recipient. KCHD, Kansas City Health Department; KDHE, Kansas Department of Health and Environment.

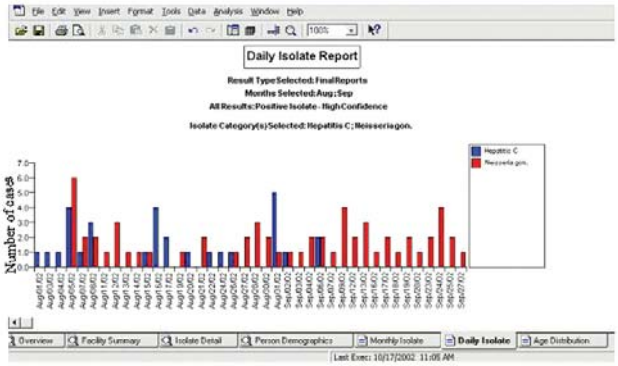

Figure 3. Example of a pathogen-trending report showing the trends for a user-selected set of pathogens. Other reports include facility summaries, detailed-line listings, and age trends.

Jurisdictional filters were applied to deliver appropriate data to the Kansas Department of Health and Environment (these data will be analyzed separately).

We evaluated reports received through both conventional and electronic reporting between March 29, 2002, and September 2, 2002, for data completeness and timeliness. Disease reporting in Kansas City requires that public health officials know the name of the testing facility and the patient's age, date of birth, race, sex, address, and telephone number. As each conventional or electronic report was received, KCHD documented whether each required data element was provided and documented the date on which conventional reports were received. Timeliness was determined by comparing this date with the date that a reportable pathogen was first posted to data clearinghouse.

Reports received only through electronic means were evaluated to confirm that they satisfied KCHD reporting criteria. This review included confirmation of appropriate jurisdictional concerns, origin of isolate (appropriate body site), and exclusion of false-positive results.

Geographic information system maps, showing the residential zip code of patients with reportable isolates, were delivered to KCHD by using ArcIMS (ESRI, Redlands, CA). Users could select a pathogen and observe the zip codes of patients with confirmed cases. Geographic coverage maps were generated by using the zip codes of all patients whose results were evaluated by the system.

Critical isolate alerts were also built into the system. A single instance of *Bacillus anthracis*, *Coxiella burnetii*, *Yersinia pestis,* or any *Brucella* species would trigger alerts sent to the pagers of on-call public health officers and supervisors.

Results

The electronic data clearinghouse study was conducted in tandem with conventional reporting at all sites. In 2002, conventional reports to KCHD originated from laboratories (52%), infection control practitioners (34%), blood centers (6%), private physicians (4%), and other sources (4%). Personnel involved in conventional reporting were generally unaware of the electronic reporting system; their management was instructed in the dual reporting requirement. Our review of reports received through both the clearinghouse and conventional reporting identified 144 isolate reports. An additional 213 cases arrived only through electronic reporting (Table 1). We reviewed the addresses of the patient and reporting laboratory to verify that a report was in the KCHD jurisdiction. Table 1 lists the specific pathogens documented through this system and the average improvement in timeliness for each pathogen. Timeliness improved for all pathogens; the improvements for chlamydia, invasive group A streptococcal infections, and salmonellosis cases were statistically significant (Table 1). One chlamydia case arrived 20 days earlier through clearinghouse reports.

Many case reports were only received through electronic reporting (Table 1). In particular, giardiasis and hepatitis C were underreported. Sexually transmitted diseases, including chlamydia, gonorrhea, and syphilis, were also underreported.

The data clearinghouse received all cases reported by traditional means for diseases that were consistently documented by using microbiology reports. The null value precluded the use of the Chandra Sekar-Deming capture-recapture technique used to assess reporting coverage in other electronic reporting systems (8,9). Diagnostic results verifying some diseases, especially viral diseases such as hepatitis and HIV, are sometimes documented using microbiology reports but are often documented using LIS applications from which results were not evaluated by the data clearinghouse.

Demographic information about patients was provided more often through clearinghouse reports than through reports received in tandem by traditional means. This improvement was statistically significant for patient address, race, age, date of birth, and sex, as determined by McNemar's test (Table 2).

Geographic coverage was examined by using ArcIMS (ESRI) to plot the zip codes of patients whose data were evaluated by the system, independent of the result (Figure 4). The breadth of geographic coverage provided by the system extended well beyond the Kansas City metropolitan area. The system evaluated patients from all regions of Missouri and Kansas, as well as out-of-state residents.

Discussion

We evaluated a data clearinghouse approach to biosurveillance using clinical microbiologic laboratory data. Electronic reporting of communicable diseases has been tested in other communities, including Hawaii, Pittsburgh,

Table 1. Comparison of reporting times between conventional and electronic reporting and evaluation of reporting coverage

Pathogen	Average days earlier[a]	Electronic and traditional[b]	Electronic only[c]	Total reports	Reporting improvement[d] (%)
Campylobacter sp.	0.6	10	7	17	70
Chlamydia trachomatis	2.2[e]	29	81	110	279
Cryptosporidium parvum	0.0	1	-	1	-
Escherichia coli O157:H7	0.0	1	2	3	200
Giardia lamblia	0.0	1	12	13	1,200
Neisseria gonorrhoeae	0.3	50	48	98	96
Haemophilus influenzae (invasive)	3.0	3	3	6	100
Hepatitis A	0.0	1	-	1	-
Hepatitis B	0.5	4	3	7	75
Hepatitis C	3.6	5	22	27	440
Influenza	1.2	5	3	8	60
Group A streptococcal infections (invasive)	2.3[f]	7	1	8	14
Borrelia burgdorferi	1.3	4	3	7	75
Salmonella sp.	2.7[f]	14	6	20	43
Shigella sp.	0.0	2	1	3	50
Streptococcus pneumoniae (invasive, drug-resistant)	8.0	1	-	1	-
Treponema pallidum	0.4	5	21	26	420
Yersinia sp.	0.0	1	-	1	-

[a]Average days earlier was calculated by comparing the date on which the initial conventional report arrived to the date on which an electronic report was received. Only cases received by both means were used to calculate this value.
[b]Reports for these cases were received by both conventional means (mail, telephone, fax) and the laboratory information network. All reports received through traditional reporting were also received by the data clearinghouse.
[c]Reports for these cases were received only through the data clearinghouse and are not included in the counts for the "electronic and traditional means" column.
[d]Received electronically only/received through both means x 100.
[e]Significant as determined by Student t test ($p<0.05$).
[f]Significant as determined by Wilcoxon signed rank ($p<0.05$).

and Indianapolis (8–10). Unlike these earlier studies, our data clearinghouse system gathered data from nonaffiliated healthcare providers, applied a centralized data-mapping team to provide efficiency of scale, and delivered data to multiple governmental entities with jurisdictions in a region that crossed state lines.

Following the bioterrorist attack involving anthrax tainted letters during fall 2001, we developed and deployed this system rapidly. Formal design of the system began in December 2001; the system was fully operational by March 29, 2002. The use of standardized extraction scripts and a centralized data-mapping operation expedited this work. The data clearinghouse system was easy for clinicians to use because data extraction was automatic and did not require them to modify their workflow, unlike other biosurveillance systems that require users to reenter data into a Web page (12,13). This clearinghouse was also the first reported system to provide laboratory order-trending information to public health organizations.

Analysis of the data clearinghouse system adds to the evidence that electronic reporting of disease can offer substantial benefits to public health. Our finding that electronic reporting improves timeliness is consistent with reports from Hawaii and other areas (9). Notably, our system attained a significant improvement in timeliness of detection for *Salmonella* spp., as well as an improvement in underreporting of this pathogen. *Salmonella* and other pathogens tracked by the clearinghouse, including *Shigella*, *E. coli* O157:H7, *Giardia lamblia,* and *Cryptosporidium parvum* are classified by the Centers for Disease Control and Prevention as Class B bioterrorism agents: food or water safety threats. Class B agents have been used in biologic crimes, including the Dulles, Oregon, contamination of salad bars with salmonellae in which 751 people became ill (14).

We compared the completeness of the data delivered by electronic reporting to data delivered by conventional means and found improvement for every data element evaluated. Greater completeness of data delivered by electronic reports is a tangible benefit for both healthcare providers and public health workers as it reduces followup requests for additional information. Some fields, address in particular, had low values for completeness even for electronic reporting, which reflects gaps in the information in patient demographic information that is provided by physician offices to clinical laboratories.

The data clearinghouse system reduced underreporting, especially for sexually transmitted diseases and enteric

Table 2. Frequency of field completion for patient demographic and care-provider information from initial reports

	Traditional reporting (%)	Electronic reporting (%)
Facility	93	97
Address	32	79[a]
Phone	29	35
Gender	94	99[a]
Race	38	72[a]
Age	92	98
Date of birth	38	95[a]

[a]Significant change as determined by McNemar's test ($p<0.05$).

pathogens, conditions that are often underreported (3,5,15). The underreported cases originated from all of the organizations participating in the electronic reporting system (data not shown). Review of a subset of underreported cases suggested three common root causes: misinterpretation of jurisdictional guidelines, misunderstanding of reportable specimen criteria, and use of outdated reporting guidelines by healthcare providers (failure to report pathogens recently added to the guidelines, as occurred with the underreported giardiasis cases).

The data clearinghouse approach to biosurveillance and disease reporting also offers opportunities to relieve healthcare providers from managing multiple jurisdictional relationships, which can lead to underreporting. Through electronic reporting, data were reported from patients residing in Kansas whose laboratory work was performed in Kansas City to both the Department of Health and Environment (with jurisdiction based on the state of residence of the patient) and KCHD (with jurisdiction over the performing laboratory). Reducing underreporting is a critical step in building a threshold-based automated alerting infrastructure because the baseline data gathered by an electronic reporting system is more accurate than that gathered by traditional methods.

During the early stages of this work, we identified a number of confounding issues. For example, a laboratory technician entered a report with the word "No" on one line, followed by "*Bordetella pertussis*" on the next line, with the intent of negating the positive result. This type of data entry error, which led to a few false-positive results propagating into the clearinghouse during the first few weeks of operation, has also been an issue for other electronic reporting systems (8). After recognizing this issue, we added context-sensitive logic to clearly flag a reportable isolate preceded by "No" or "Not" as a potential false-positive.

We evaluated whether test orders were predictive of disease incidence. However, in the absence of major outbreaks during the period evaluated, our data were inconclusive. Surveillance of laboratory test orders (i.e., ova and parasite procedure orders) would be useful as an early warning of a water-supply contamination crises, such as that experienced by Milwaukee in 1993 when cryptosporidium contaminated the city water supply and caused >403,000 illnesses (16).

Most patients whose results were evaluated by the system resided within approximately 150 km of the Kansas City metropolitan area; however, patients from eastern Missouri, western Kansas, and other U.S. states also were evaluated by the system. The area covered by the clearinghouse crosses the state line that transects the Kansas City metropolitan area. Plans for a national public health infrastructure (e.g., the National Electronic Disease Surveillance System [NEDSS] [17]) rely on a process in which patient results are first sent to the state health departments and then forwarded to the Centers for Disease Control and Prevention, currently with identifiable information removed. Reconciling duplicate cases without such information will prove difficult. Systems such as NEDSS that manage data based on politically defined boundaries have inherent inefficiencies that could be rectified by direct reporting to a data clearinghouse. A data clearinghouse can apply jurisdictional filters that control the distribution of reports, while also offering the opportunity to perform rapid analysis of trends across politically defined boundaries.

We considered issues that have proven to be challenging for other electronic reporting projects. In particular, the use of nonstandardized data, subject to errors in either database design or during data entry, created challenges. Overall, we found that the data clearinghouse approach to biosurveillance offers many benefits, including ease and speed of implementation, improved timeliness and completeness of data, efficiency of scale from a central data-mapping operation, and the ability to deliver data to multiple jurisdictions.

Figure 4. Number of patient encounters evaluated by the data clearinghouse system for potential reportable events per zip code.

Acknowledgments

We thank Lesha Dennis, Gary Dickerson, Suzanne Fiske, Andrew Friede, Jody Gosch, Kim Hlobik, and Karen Miscavish; the Information Technology staff at Health Midwest; St. Luke's Shawnee Mission, Truman; Sisters of Mercy; and the Kansas City Health Department.

This project was funded independently by Cerner Corporation and the Kansas City Health Department. Cerner Corporation has a financial interest in the HealthSentry clearinghouse system.

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Environmental Risk and Meningitis Epidemics in Africa

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Epidemics of meningococcal meningitis occur in areas with particular environmental characteristics. We present evidence that the relationship between the environment and the location of these epidemics is quantifiable and propose a model based on environmental variables to identify regions at risk for meningitis epidemics. These findings, which have substantial implications for directing surveillance activities and health policy, provide a basis for monitoring the impact of climate variability and environmental change on epidemic occurrence in Africa.

Epidemics of meningitis occur worldwide. However, the "meningitis belt" of Africa's Sahel region has the greatest incidence of the disease, with large epidemics attributed to predominantly group A meningococci; the endemic levels found in this region would be considered epidemic elsewhere. Although factors predisposing populations to meningitis epidemics are poorly understood, population susceptibility, introduction of new strains, poor living conditions, and concurrent infections have all been implicated. Epidemics occur throughout Africa in the dry season, coincide with periods of very low humidity and dusty conditions, and disappear with the onset of the rains, suggesting that these environmental factors may also play an important role in the occurrence of the disease (1–3).

Lapeyssonnie (4) observed in 1963 that epidemics largely occurred in a semi-arid zone south from the Sahara, with 300–1,100 mm mean annual rainfall, and Cheesbrough et al. (5) suggested in 1995 that areas that are humid throughout the year have low disease rates. In West Africa, Waddy (1958) described an area that suffered epidemics as having "… only one definable frontier, the junction of the forest…with the savanna…, when there is an abrupt change from a permanently humid climate to one with a severe dry season" (6). Epidemics have been rarely reported from the humid forested or coastal regions, even when neighboring areas are severely affected.

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The timing of future outbreaks is unpredictable; tools that identify the key environmental factors associated with areas prone to meningitis epidemics would help us to understand the basis for these outbreaks and eventually optimize prevention and control activities. We describe a model that predicts the probability, based on environmental information, of an area experiencing an epidemic of meningitis.

Methods

Epidemiologic Data

Details of all known meningitis epidemics occurring before 2000 in countries comprising continental Africa were compiled from information documented in the published literature and unpublished institutional reports at the end of June 2001. All epidemics reported in the medical literature were identified in PubMed's online database of medical literature (United States National Library of Medicine, available from: URL: http://www.ncbi.nlm.nih.gov/PubMed/) through manual searches and by cross-referencing publications. We obtained unpublished information from Web searches or directly from international and national organizations involved in disease control and humanitarian aid (1).

Epidemics reported at the provincial (second administrative level) and district level were located by using administrative boundaries available from the U.S. Geological Survey EROS Data Centre Africa Data Dissemination Service, Sioux Falls, SD (available from: URL: http://edcintl.cr.usgs.gov/adds/). Locations of villages or towns were verified by using the National Imagery and Mapping Agency's GEONet gazetteer (available from: URL: http://www.nima.mil/), historical reference atlases and maps contained in the original reports, and were mapped to the current administrative boundaries by using ArcView 3.1 geographic information system (GIS) (ESRI, Redlands, CA). We assumed that events reported at the provincial level affected all constituent districts and excluded epidemics reported only at the national level.

Events affecting parts of any one country in one epidemic season were considered as one epidemic. We then classified the 3,281 districts of Africa as ever or never having experienced a documented epidemic of meningitis. In this analysis, no attempt was made to distinguish between epidemics of different scales.

Environmental Data

Environmental information for the African continent was obtained from a variety of sources. In this analysis, we restricted data to variables available in the public domain with digital grid–based uniform continental coverage, which we considered important. Variables included monthly means (long-term averages) of absolute humidity (7), absorbing aerosols (dust) and rainfall, and land-surface maps of land-cover type and population density (Table 1). All data grids were incorporated into the GIS.

To collect the seasonal variation in climate while reducing the number of explanatory variables without loss of information, we reprocessed the monthly means of each variable to create a single surface comprising categories representing unique seasonal profiles. This reprocessing involved submitting the monthly means to a principal components analysis followed by a clustering procedure in which we grouped regions with a similar seasonal profile using ADDAPIX software (version 2.05, S. Griguolo, University of Venice, Italy, available from: URL: http://metart.fao.org/T_I/GBR/Tools/Eaddapix.htm). This software performs spatial and temporal analyses of time-series data in continuous grid-based surfaces (8). The use of seasonal profiles for describing the climate of an area is widely used in crop monitoring in agriculture and has been used in modeling malaria prevalence in Gambia (9). The profile surfaces were then imported into ArcView 3.1 (ESRI). The seasonal absolute humidity profile is shown in Figure 1.

For every district, a value was extracted in GIS representing those grid cells of each profile surface contained within the district boundary. These values comprised the most common seasonal profile class for each variable (absolute humidity, dust, and rainfall), the most common land-cover type, and the geometric mean population density.

Analysis

A logistic regression analysis was used to identify associations between a district ever or never having experienced an epidemic and the environment by using SPSS 11.0 software (SPSS Inc., Chicago, IL). Explanatory variables were first investigated individually and then entered stepwise into a forward conditional multiple regression analysis. Because of the need to reduce colinearity, we did not analyze environmental variables with similar geographic distributions together in the same model. The final model was based on the simplest approach and a combination of variables that best predicted the distribution of epidemics. This model was created with and without weighting for the inverse size of the district to assign less weight to larger districts, which may have been more prone to ecologic variation and therefore inadequately represented by a single value. The probability of each district ever having had an epidemic was predicted by using the model. These probabilities were grouped into risk categories and mapped, and the estimated total population was extracted in GIS derived from population density forecasts (U.S. Geological Survey, 1990).

The sensitivity and specificity of the model were assessed by examining the agreement between predicted and observed epidemic experience by using a receiver-operator characteristics (ROC) curve to select the optimal probability cutoff values on which predictions are based (10). The dataset was then split at random into two parts containing approximately 60% and 40% of the 3,281 districts. The model was recreated with the 60% dataset, using the same variables as above and used to predict the risk for epidemics in the remaining 40% (the validation set). We repeated this process 10 times and compared the mean sensitivity and specificity of the validation set with the model

Table 1. Characteristics of the environmental variables

Variable	Temporal resolution	Time period	Resolution of grid squares
Interpolated meteorologic station data[a]			
Average daily mean absolute humidity	Mean monthly	1961–1990	0.5° lat x 0.5° long (nominal 50 km)
Average daily rainfall	Mean monthly	1961–1990	0.5° lat x 0.5° long (nominal 50 km)
Remotely sensed satellite data[b]			
Average daily aerosol index (dust)	Mean monthly	1980–1999	1.0° lat x 1.25° long (nominal 100 km)
Digital maps			
Land-cover type[c]		1992–1993	1 x 1 km
Population density[d]		1990	0.042° lat x 0.042° long (nominal 4 km)

[a]Mean monthly climate averages (1961–1990) for absolute humidity and rainfall (the former derived from vapor pressure and mean temperature [7]) were obtained from the Climate Research Unit, University of East Anglia, UK (available from: URL: http://ipcc-ddc.cru.uea.ac.uk/).
[b]Dust was obtained as monthly aerosol index coverages from the U.S. National Aeronautics and Space Administration (NASA), Goddard Space Flight Center, Maryland (available from: URL: http://www.gsfc.nasa.gov/), excluding the period May 1993 to June 1996 for which data are not available.
[c]Land-cover type was obtained from the USGS NASA PATHFINDER 1km project (available from: URL: http://edcdaac.usgs.gov/1KM/1kmhomepage.html/).
[d]U.S. Geologic Survey 1990 population density forecasts (available from: URL: http://grid2.cr.usgs.gov/globalpop/africa/).

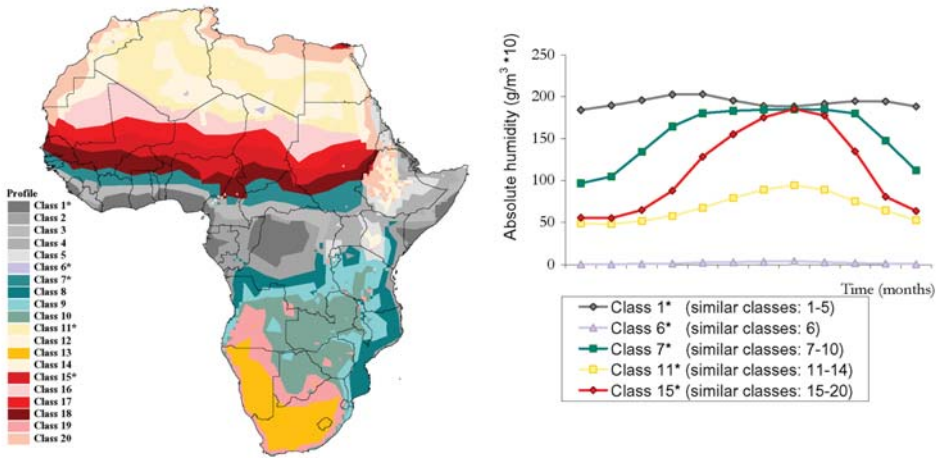

Figure 1. Ecologic variation in the seasonal profile of absolute humidity. a) spatial variation in profile class; b) representative profile class.

derived from the entire dataset. Residuals resulting from the differences between the observed and predicted risks were also calculated and mapped to establish whether errors in the model were randomly distributed, thereby supporting the validity of the model throughout the continent.

Results

The earliest documented meningitis outbreak in Africa affected a French garrison in 1841 in Algiers (11), and ≥425 epidemics were documented at the subnational level for the next 158 years. These epidemics affected at least 1,231 (38%) of the 3,281 continental districts. Supporting our earlier findings, epidemics were not evenly distributed across the continent, instead affecting mainly districts in the Sahel and south of this region and extending from northern Uganda and the eastern part of Democratic Republic of Congo, through the Great Lakes and the Rift Valley regions to Malawi and northeastern Mozambique, and from northeastern Mozambique west and south to include other parts of southern Africa (Figure 2a) (1).

Absolute humidity, dust and rainfall profiles, land-cover type, and population densities were independently associated with the location of epidemics. However, we found that absolute humidity profile and land-cover type were the best predictors in the final multivariable model. Certain dust profiles and population density made only marginal difference to the performance of the model and were excluded to maintain simplicity. In addition, absolute humidity and rainfall profiles predicted similar risks in geographic locations in north and west Africa, but a model including the former performed better for the entire continent than one including rainfall; for this reason, we kept absolute humidity as a variable. Weighting for district size did not improve the performance of the model and was discarded.

The model, which is based on the absolute humidity profile and land-cover type, is described in terms of its baseline characteristics, estimated coefficients, standard errors, and contribution of the variables in Table 2. The risk map for epidemic experience in Africa derived from this model is presented in Figure 2b. The most important

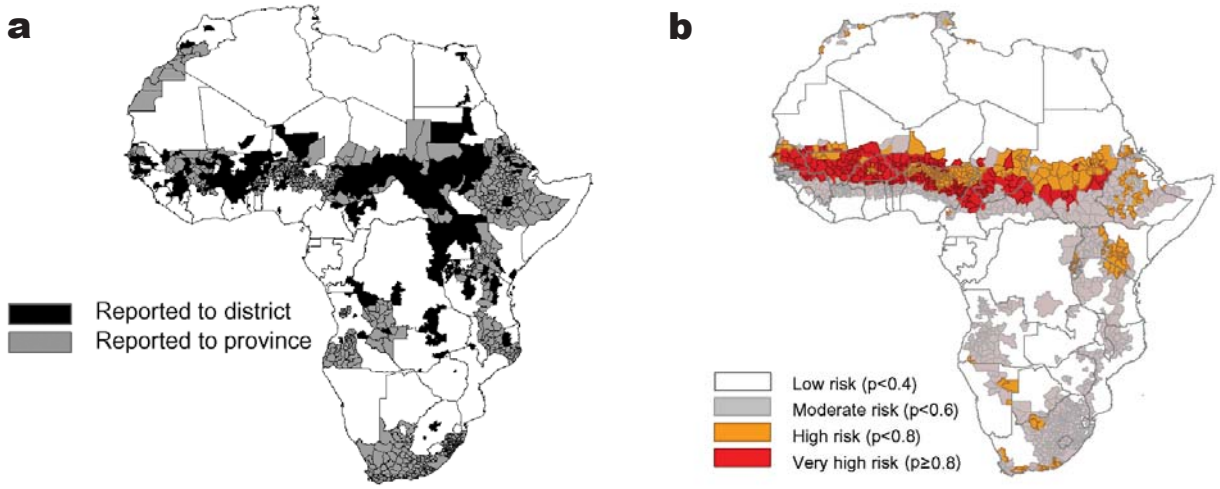

Figure 2. Distribution of districts where epidemics of meningococcal meningitis are likely to occur. a) observed distribution of meningitis epidemics (1841–1999). b) predicted probabilty of epidemic experience based on environmental variables.

Table 2. Baseline characteristics, estimated coefficients, and standard errors for the model

	Epidemic experience (n districts)[a]		Multivariable analysis	
Variable	Ever (%)	Never	β	SE
Absolute humidity profile[b]				
Class 1	15 (3)	527	Reference	
Class 2	9 (6)	153	0.59	0.44
Class 3	19 (8)	228	1.00[c]	0.36
Class 4	43 (47)	48	3.48[c]	0.35
Class 5	143 (61)	93	3.97[c]	0.31
Class 6	0 (0)	2	–2.44	15.73
Class 7	118 (47)	132	3.29[c]	0.30
Class 8	40 (28)	102	2.40[c]	0.33
Class 9	106 (48)	117	3.31[c]	0.31
Class 10	46 (22)	161	2.12[c]	0.32
Class 11	16 (29)	39	3.34[c]	0.43
Class 12	1 (11)	8	2.17[d]	1.13
Class 13	90 (48)	97	3.30[c]	0.32
Class 14	44 (52)	40	4.00[c]	0.36
Class 15	178 (74)	64	4.46[c]	0.32
Class 16	7 (37)	12	3.61[c]	0.59
Class 17	25 (54)	21	3.99[c]	0.43
Class 18	181 (80)	46	4.82[c]	0.32
Class 19	105 (56)	84	3.60[c]	0.31
Class 20	46 (39)	73	3.45[c]	0.35
Land-cover type				
Savanna	646 (39)	1006	Reference	
Dryland cropland/pasture	44 (39)	68	–0.38	0.23
Irrigated cropland/pasture	0 (0)	9	–7.02	7.14
Cropland/grassland mosaic	198 (53)	174	–0.07	0.14
Cropland/woodland mosaic	4 (4)	105	–1.97[c]	0.56
Grassland	123 (67)	61	0.36	0.19
Shrubland	66 (34)	127	–0.52[c]	0.19
Urban	3 (43)	4	0.32	0.85
Broadleaf deciduous forest	44 (39)	68	0.17	0.23
Evergreen broadleaf forest	37 (13)	248	0.07	0.24
Water bodies	20 (38)	33	–0.07	0.33
Forest wetland	0 (0)	24	–2.82	4.55
Barren/sparsely vegetated	46 (28)	120	–1.07[c]	0.24
Variable removed[e]	Log likelihood	Change in –2 log likelihood	df	Significance of change
Absolute humidity profile	–2045.937	877.963	19	<0.001
Land-cover type	–1642.564	71.217	12	<0.001

[a]Excludes five districts for which environmental data were unavailable (in Sinai, Egypt, and Pemba, Tanzania).
[b] See Figure 1 for description of profile classes.
[c]p value ≤0.01.
[d]p value ≤0.05.
[e]Model if term removed.

factor associated with the distribution of epidemics was humidity. Areas without a marked distinction between wet and dry seasons were less likely to have had epidemics than those with contrasting seasons. The areas without distinction between wet and dry seasons include deserts and the humid parts of coastal and central Africa, much of which are forested, and the areas with contrasting seasons comprise the semiarid savannah and grasslands of the Sahel and east and southern Africa. Surface maps of Africa demonstrated a close correspondence between humidity and land-cover types in these regions. The model also showed that, having accounted for the effects of humidity, sparsely vegetated and barren regions, areas of woodland mosaic, and shrub land were less likely than other regions to have ever had an epidemic. The Sahel, which has a prolonged dry season with low humidity was identified as the area with the greatest risk ($p>0.6$). Peripheral regions along its southern borders, where the dry season is shorter and less extreme, carry a moderate risk ($p>0.4$). The peripheral region extends from southern Sudan and Ethiopia to the Great Lakes and Rift Valley regions and parts of southern Africa peripheral to desert areas.

The ROC curve used to describe the performance of the model in terms of its sensitivity and specificity at various cutoffs, and its overall accuracy independent of a single probability cutoff is shown in Figure 3. The model can discriminate between districts that have experienced epidemics and those that have never been affected. When the values for randomly selected districts were entered into the model, the epidemic risk assigned was higher for districts with epidemics than for those without in 82% of the cases. The areas identified by the model coincide to a large extent with the areas observed to have experienced epidemics. By using a probability cutoff value of ≥0.4 for predicting epidemic experience, the model had a sensitivity and specificity of 83% and 67%, respectively; these statistics were confirmed in the validation process (Table 3). The map of residual risk unaccounted for by the model had a random distribution, as expected for a model that worked well across the continent (not shown). According to the model, 7% of the population of Africa live in very high-risk areas, 17% in high, and 27% in moderate (based on 1990 estimates), corresponding to 44, 102, and 162 million people, respectively (Table 4).

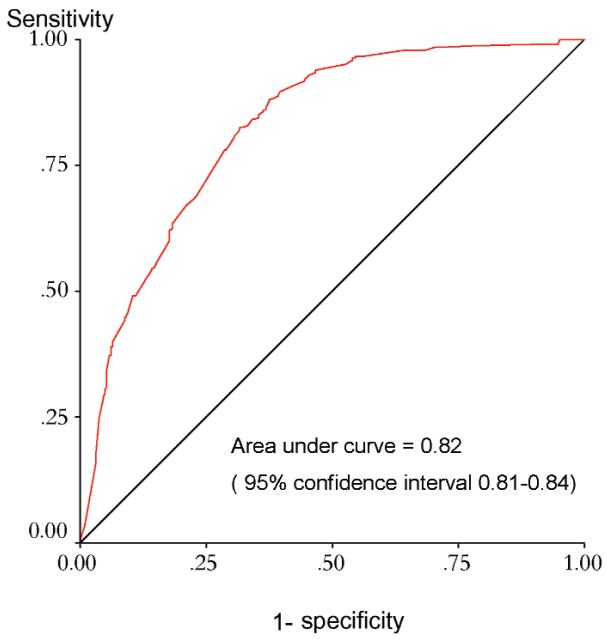

Figure 3. Receiver-operator characteristics curve for the model.

Discussion

This study represents a comprehensive and detailed description of the spatial distribution of meningitis epidemics at the district level in Africa and the first attempt to develop a spatial forecasting model for meningitis epidemics on the basis of the environmental characteristics of the continent considered a priori to be related to the spatial distribution of epidemics. The data have limitations that need to be considered for the proper interpretation of the models. For example, census data for Africa have limited accuracy (12), variables derived through remote sensing may only partially capture surface conditions, and data from meteorologic stations in Africa are often incomplete (13–15). The epidemiologic data span more than a century whereas the environmental and population data are relatively recent. In addition, some epidemics were likely never reported, and small outbreaks and those occurring in the 19th and early 20th centuries are likely to be disproportionately underrepresented. Problems in defining epidemics exist as well, since most reports lack clear or internationally recognized criteria; we had to accept the perception of an increased incidence that prompted outbreak reports. Moreover, the aggregation of local level statistics to an often larger and somewhat arbitrary district level, discrepancies between where people become ill and the location of notifying health facilities, and changes to district boundaries over time may each have resulted in potential loss of specificity. Despite these limitations, major outbreaks were unlikely to have gone completely unreported, and the long-term cumulative distribution of events is likely not misrepresented on a pancontinental scale. While population densities in Africa have increased greatly during the last 150 years and substantial land-use change (particularly in West Africa) is known to have occurred, the model was still able to identify the meningitis belt and areas previously described at risk beyond the Sahel (2–5,16); reports of epidemics occurring since 1999 have coincided with this description (available from: URL: http://www.who.int/disease-outbreak-news/).

The model also has its limitations. We are aware of those imposed on the analysis by spatial relationships in the data, which indicate that the importance of regression effects may be overstated (17). No perfect model exists, but one can be developed that is based on the simplest approach and combination of variables that can be used to distinguish between areas with high and low risk of epidemic experience. We were restricted by the limited avail-

Table 3. Performance of the model for predicting epidemic experience at the district level[a]

Epidemic experience	Observed		
Predicted	Ever	Never	Total districts
Ever	1,022	682	1,704
Never	209	1,363	1,572
Total districts	1,231	2,045	3,276
Model	**Sensitivity % (95% CI)**		**Specificity % (95% CI)**
Final (100%)	83 (81 to 85)		67 (65 to 69)
Mean validation (40%) (n=1a)	84 (80 to 87)		65 (62 to 69)

[a]Excludes five districts for which environmental data were unavailable
CI, confidence interval.

Table 4. Population residing in districts at risk for meningitis epidemics in Africa

Risk	No. of districts	1990 population in millions (%)
Low	1,572	291 (49)
Moderate	971	162 (27)
High	482	102 (17)
Very high	251	44 (7)
Not known	5	1 (<1)
Total	3,281	600

ability of suitable datasets and chose the simplest combination of variables that best predicted outcome. While this choice is likely to have oversimplified the association between meningitis epidemics and the environment, the model has the advantage of using data that are available in the public domain and, being based on a simple combination of variables, are an important basis on which to develop research and future operational applications in resource-limited settings.

The analysis indicates not only that absolute humidity profiles and land-cover types can be used to distinguish between areas with high and low risk of epidemics but also that population density and dust may also be implicated. The incidence of meningococcal disease has previously been correlated with dry and dusty conditions in tropical and temperate climates (18–21). Humidity and land cover were included in the final model for statistical reasons, but dust and population density still have an independent effect and may be important in determining epidemic occurrence (22). The potential role of dust in precipitating epidemics is particularly interesting since dustiness in the meningitis belt has increased dramatically since the Sahelian droughts of the 1970s and 1980s. However, how environmental variables interact is unclear and remains the subject of extensive climatologic research. Furthermore, we did not take into account the effect of other nonenvironmental factors likely to be related to epidemics, such as population movement, vaccination coverage, and recent epidemics in the area. A combination of conditions is likely to be necessary for an epidemic to occur, and these nonenvironmental variables are likely to have additional predictive potential and should be considered in further studies.

Risk maps of vector-borne diseases in Africa based on environmental data have received considerable attention in recent years and are tools with public health potential (10,15). The mechanisms by which environmental factors influence meningitis epidemics in Africa are unclear (3). Areas within the traditional meningitis belt and beyond, however, are environmentally susceptible to epidemics with potentially large populations at risk; markers such as absolute humidity and dust profiles, land-cover type, and population density are independent predictors of these areas. In addition, rainfall and dust are predictors in some, but not all regions, and the potential to develop region-specific models that could be more sensitive within given ecologic zones warrants further study. Our findings could facilitate the development of models to identify regions with increased vulnerability to epidemics in the future and provide a basis for monitoring the impact of climate variability and environmental change on epidemic occurrence in Africa.

Acknowledgments

We thank Jay Herman for facilitating access to the aerosol data used in the analyses, Ian Hastings for statistical advice, and all persons and organizations that have contributed to the Meningitis Forecasting Project for their support and collaboration.

Financial support was received from the Meningitis Research Foundation (UK), the U.S. National Oceanic and Atmospheric Administration Office of Global Programs, and Médecins Sans Frontières.

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Severe Acute Respiratory Syndrome: Lessons from Singapore

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An outbreak of severe acute respiratory syndrome (SARS) occurred in Singapore in March 2003. To illustrate the problems in diagnosing and containing SARS in the hospital, we describe a case series and highlight changes in triage and infection control practices that have resulted. By implementing these changes, we have stopped the nosocomial transmission of the virus.

An outbreak of severe acute respiratory syndrome (SARS) was first recognized in Singapore on March 12, 2003. The index patient was hospitalized at Tan Tock Seng Hospital, which has since become the country's designated SARS hospital. The patient infected 20 other people (including patients and healthcare workers), who subsequently became the sources for secondary spread of the infection (1). As of June 12, 2003, a total of 206 cases and 31 deaths attributed to SARS had been reported in Singapore.

We describe the important lessons learned during the triage and containment of SARS at the National University Hospital, Singapore. Both involved expanding isolation criteria to include all patients with undifferentiated fever (even in the absence of respiratory symptoms or chest x-ray changes), improving contact-tracing methods, enforcing the use of fit-tested personal protective equipment in all patient-care areas, avoiding aerosol-generating procedures, and carefully monitoring all healthcare workers for fever or respiratory symptoms. We also highlight the impact of these measures on preventing the entry and nosocomial spread of infection.

The Study

From March 13 to May 5, 2003, we identified all epidemiologically linked patients whose disease met the Centers for Disease Control and Prevention's case definition of SARS issued on April 29, 2003 (2). Initial investigations included a complete blood count (with a differential count), serum biochemical measurements (including electrolytes, renal and liver function values, creatine kinase, and lactate dehydrogenase), and a chest x-ray. Since the cause of the virus was not known at the onset of the outbreak, routine microbiologic cultures of sputum, urine, and blood were done to rule out common bacterial causes of pneumonia. In addition, mycoplasma serology and urine *Legionella* antigen testing were carried out. When reverse transcriptase–polymerase chain reaction (RT-PCR) kits for coronavirus detection became available, later patients also provided samples for RT-PCR.

Probable SARS was diagnosed in 14 patients and healthcare workers at National University Hospital. The median age of the patients (five men and nine women) was 58 years (range 21–84). Detailed patient characteristics, including background, medical histories, symptoms, and signs, are shown in Tables 1 and 2.

Case Histories

Case 2

A woman 43 years of age was admitted to the hospital on March 23; she had had fever, headache, vomiting, and coughing for 7 days and diarrhea on the first day of her illness, which spontaneously resolved. She reported no SARS contacts. The patient was admitted to an isolation room with the diagnosis of community-acquired pneumonia, but her condition rapidly deteriorated. She was transferred to the intensive care unit (ICU), where she died on March 31. On day 3 of her hospital stay, healthcare workers discovered that she had previously visited a friend with hepatitis at the Tan Tock Seng Hospital. Two unidentified SARS patients had been on that hospital ward.

Case 3

Case-patient 3 was an ICU physician who performed a bronchoscopy on case-patient 2 on March 26. This procedure was performed in a negative-pressure room with gloves, gown, and an N95 mask. He became ill with fever, headache, and myalgia on March 29. His initial chest x-ray was clear, and his fever resolved transiently for 30 hours before recurring. Subsequent chest x-rays showed right lower-lobe infiltrates that went on to involve both lung fields. He eventually required intubation and ventilatory support in the ICU. He was successfully extubated and has since been discharged.

Case 6

A man 63 years of age with coronary artery disease was admitted to the hospital on April 8 after he reported dizziness and shortness of breath. He had seen his general practitioner 2 days earlier with complaints of rhinorrhea, cough,

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Table 1. Admitting clinical characteristics of patients 1 through 7 with severe acute respiratory syndrome

Characteristic	Patient 1	2	3	4	5	6	7
Sex	F	F	M	F	F	M	M
Age	71	43	40	78	53	63	84
Source of virus	Community	Community	HCW	Community	Community	Community	Inpatient
Admission diagnosis	Meningitis	Pneumonia	Suspect SARS	Interstitial lung disease	Pneumonia	Congestive heart failure	Pneumonia SARS contact
Co-illnesses	IHD; DM; hypothyroidism	Hypertension		IHD, ypertension; chronic renal failure; CTD on steroids		IHD Hypertension	IHD Cerebrovascular disease
Symptoms							
Cough	-	+	+	-	+	+	-
Dyspnea	-	+	-	+	+	-	+
Rhinorrhea	-	-	-	-	-	+	-
Sore throat	-	+	-	-	-	-	-
Headache	-	+	+	-	-	-	-
Myalgia	-	-	+	-	-	+	-
Fever	+	+	+	-	+	+	+
Others	Confusion	Vomiting, diarrhea				Dizziness	
Temp on admission (°C)	38.7	38.9	36.8	36.0	39.6	35.1	38.0
Physical signs							
Crackles	+	+	+	+	+	+	+
Chest x-ray findings	Right basal infiltrates	Bibasilar infiltrates	Right basal infiltrates	Right basal infiltrates	Bilateral infiltrates	Bilateral infiltrates	Right upper lobe infiltrates
RT-PCR	ND	ND	Positive	ND	Positive	Positive	ND
Outcome	Survived	Died	Survived	Died	Died	Died	Died

[a]F, female; M, male; HCW, healthcare worker; SARS, severe acute respiratory syndrome; IHD, ischemic heart disease; DM, diabetes mellitus; CTD, connective tissue disease; RT-PCR, reverse transcriptase polymerase chain reaction; ND, not done.

and myalgia. He had a documented temperature of 37.7°C at the physician's office. On admission he was afebrile, and his chest x-ray showed cardiomegaly with bilateral lung infiltrates. He was admitted to the general medical ward with probable congestive heart failure. However, a transthoracic echocardiogram showed a normal ejection fraction. Within 12 hours, he became critically ill and was transferred to the ICU, where he was intubated. His repeat chest x-ray showed worsening bilateral infiltrates consistent with acute respiratory distress syndrome. The patient had visited an ill brother at the Singapore General Hospital (hitherto a SARS-free hospital). The brother had previously been in Tan Tock Seng Hospital, on March 9–31, and was subsequently identified as the index case-patient for the outbreak at the Singapore General Hospital (3). Case-patient 6 went on to infect 15 other people.

Case 11

Case-patient 11 was the on-call physician who assessed case-patient 6 and transferred him to the ICU. She had worn a gown, gloves, and N95 mask, despite no requirement to do so on the general ward at that time. She had fever, headache, and myalgia 3 days later. Her chest x-ray on admission was clear. Respiratory and chest x-ray changes occurred on day 5 of illness; the patient was discharged after 12 days.

Course of Illness

Most of the patients had a prodrome of fevers and myalgias with no respiratory or chest x-ray changes until several days later. Their illnesses ran a steady course, lasting a median of 11 days. In case-patient 3, the illness exhibited a biphasic pattern with a brief resolution of fever, followed by the return of high temperature and progression of respiratory and chest x-ray changes. A subset of these patients had a fulminant course with rapidly progressing respiratory failure requiring intubation and mechanical ventilation.

Hematologic and Biochemical Findings

The hematologic and biochemical findings of the case-patients on admission are summarized in Tables 3 and 4. Leukocyte count was normal in nine case-patients. Mild leukopenia was observed in one case-patient, and leukocytosis was observed in another. Lymphopenia (defined as $<1.50 \times 10^{-9}$/L) was observed in all patients. We found mild thrombocytopenia in two case-patients. The lactate dehydrogenase levels were elevated in eight case-patients. The C-reactive protein was elevated in 7 of 11 case-patients, and the procalcitonin was raised in four of five case-patients.

Radiologic Changes

We saw a variety of chest x-ray changes in these patients (Tables 3 and 4). The primary abnormalities were

Table 2. Admitting clinical characteristics of patients 8 through 14 with severe acute respiratory syndrome

	Patient						
Characteristic	8	9	10	11	12	13	14
Sex	M	F	M	F	F	F	F
Age	63	74	72	28	24	28	21
Source	Inpatient	Inpatient	Inpatient	HCW	HCW	HCW	HCW
Admission diagnosis	Pneumonia; SARS contact	*Escherichia coli* UTI; SARS contact	Pneumonia. SARS contact	Suspect SARS	Suspect SARS	Suspect SARS	Suspect SARS
Co-illnesses	IHD; DM; hypertension	IHD; DM; hypertension; cerebrovascular disease	Dilated cardiomyopathy; hypertension; chronic renal failure	None	None	None	None
Symptoms							
Cough	-	-	-	-	-	-	-
Dyspnea	-	-	-	-	-	-	-
Rhinorrhea	-	-	-	-	-	-	-
Sore throat	-	-	-	-	+	-	-
Headache	-	-	-	+	-	-	+
Myalgia	-	-	-	+	+	+	+
Fever	+	+	+	+	+	+	+
Others							
Temp. on admission (°C)	38.5	38.0	38.5	38.2	38.0	38.5	38.8
Physical signs							
Crackles	+	-	+	-	-	-	-
Chest x-ray findings	Right basil infiltrates	Bibasilar infiltrates	Right basal infiltrates	Right basal infiltrates	Right basil infiltrates	Right upper lobe infiltrates	Left upper lobe infiltrates
RT-PCR	ND	Positive	Positive	Positive	Positive	Positive	Positive
Outcome	Survived	Died	Survived	Survived	Survived	Survived	Survived

[a]M, male; F, female; HCW, healthcare worker; SARS, severe acute respiratory syndrome; UTI, urinary tract infection; IHD, ischemic heart disease; DM, diabetes mellitus; RT-PCR, reverse transcriptase polymerase chain reaction; temp., temperature; ND, not done.

patchy unilateral or bilateral consolidation. Opacities were predominantly in the lower lung zones in most patients. Three patients had upper lobe infiltrates. However, five of the patients were admitted to the hospital with normal chest x-ray results.

Conclusions

On March 18, 2003, our emergency department began screening all febrile patients with respiratory complaints for possible SARS (2). All suspected SARS patients were admitted to a negative-pressure isolation room for monitoring. Probable SARS case-patients were transferred to Tan Tock Seng Hospital for further management to keep the hospital free of the SARS virus.

The varied clinical signs and symptoms of SARS have limited the success of such a triage system (4–8). We have observed that current diagnostic guidelines may not be sufficiently sensitive for assessing patients before admission to hospital. For example, although a temperature of >38°C is part of the diagnostic criteria (2), this symptom was notably absent in two patients. The lack of a fever in case-patient 4 may have been the result of long-term steroid use. Case-patient 6 never had a documented temperature ≥37.7°C and was hypothermic throughout his hospitalization. In addition, a subset of patients may have fever and myalgias without respiratory complaints or chest x-ray

Table 3. Laboratory data for patients 1 through 7 admitted with severe acute respiratory syndrome

Patient	1	2	3	4	5	6	7
Hemoglobin (g/dL)	**10.9**	12.5	13.4	14.1	12.0	16.3	12.1
Leukocyte count ($X10^{-9}$/L)	4.52	**19.34**	5.3	**14.65**	5.41	9.29	10.14
Lymphocyte count ($X10^{-9}$/L)	**0.78**	**0.94**	**0.71**	**1.02**	**0.53**	**0.63**	**0.21**
Platelet count ($X10^{-9}$/L)	**117**	149	189	180	176	136	**82**
ALT (U/L)	55	37	23	22	**76**	35	**210**
AST (U/L)	**65**	**108**	39	**102**	**121**	**92**	**58**
CK	290	183	213	86	**1,045**	124	**499**
LDH	**747**	**2,513**	**518**[b]	**1,032**	**2,248**	**2,015**	**955**
C-reactive protein	**5.2**	**34.0**	**141.4**	**27.4**	ND	ND	**7.4**
Procalcitonin	0.14	**1.45**	ND	**1.27**	ND	**15.21**	ND

[a]ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; LDH, lactate dehydrogenase; ND, not done; values in boldface are abnormal.
[b]Normal value for LDH at this laboratory was <500.

Table 4. Laboratory data for patients 8 through 14 admitted with severe acute respiratory syndrome

Patient	8	9	10	11	12	13	14
Hemoglobin (g/dL)	15.0	8.4	11.2	12.5	14.3	13.2	13.5
Leukocyte count ($X10^{-9}$/L)	3.73	7.2	4.94	6.3	3.6	6.2	6.76
Lymphocyte count ($X10^{-9}$/L)	0.83	1.34	1.04	0.30	0.84	1.13	0.97
Platelet count ($X10^{-9}$/L)	121	163	167	231	176	184	240
ALT (U/L)	51	31	20	10	15	19	8
AST (U/L)	68	52	19	21	21	21	25
CK	35	192	73	68	85	88	56
LDH	685	1,034	390	280	275	319	696
C-reactive protein	3.1	4.3	0.9	<0.7	<0.7	<0.7	<0.7
Procalcitonin	ND	1.27	ND	ND	ND	ND	ND

[a]ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; LDH, lactate dehydrogenase; ND, not done.

changes until later (cases 3 and 11). Booth et al., in a retrospective analysis of 144 patients with SARS, reported that 11% of patients had no respiratory symptoms and would not meet current criteria for SARS, despite having fever, contact history, and chest x-ray infiltrates (9). Rainer et al., in a study of 515 patients attending a SARS screening clinic, found that current World Health Organization guidelines, which emphasize respiratory tract symptoms, had a low sensitivity of 26% (10). We expanded our isolation criteria to include all patients with undifferentiated febrile illness and chest x-ray infiltrates with or without fever or respiratory symptoms until an alternative diagnosis is made, or until they defervesce and serial chest x-ray findings are normal.

The nonspecific symptoms of this illness often require establishing a history of contact with a SARS patient as a critical clue to the diagnosis (2). However, a contact history may not be forthcoming at the initial interview. In Singapore, SARS is an imported infection in which the epidemiology remains well defined with clear lines of secondary spread in the hospitals and community (3). Emphasis is placed on using local epidemiologic clues to elicit a history of visits or previous admissions to Tan Tock Seng Hospital or other healthcare facilities and to inquire whether any family members are ill or hospitalized. Patients without a contact history are still isolated if the clinical suspicion is high. A national computerized database of SARS patients and their contacts has been established to assist with this process. An epidemiology team is also used to perform more exhaustive contact tracing and liaisons with other healthcare facilities. This team also monitors and investigates any clusters of pneumonias within our hospital. Suspected case-patients are then immediately transferred to the isolation area for further investigation.

Our initial infection-control policy first required staff working in the emergency department, ICU, and isolation rooms to wear full personal protection equipment (PPE), which included an N95 mask, disposable gloves, and long-sleeve gowns. Virus transmission is likely due to droplet infection, but fecal-oral transmission has also been reported (11). The role of fomites has yet to be defined; preliminary data suggest that the virus can remain viable at room temperature for at least 2 days. Recognizing that patients with unsuspected cases of SARS could slip through our triage system onto the general wards (see case 6), all hospital staff with direct patient contact were required to wear full PPE beginning April 8. In addition, all patients are now examined with the use of dedicated equipment.

Despite the use of PPE, two of our physicians were infected with the virus. The first physician performed an invasive procedure (bronchoscopy) on case-patient 2. The outlet port of the patient's ventilator was later discovered to have malfunctioned during the procedure, exposing the physician to a large jet of exhaled air. Reports of protected healthcare workers becoming infected during intubation of SARS patients have also emerged from Canada (12). The recommendations on the use of PPE are likely insufficient for procedures that may promote aerosolization of respiratory secretions (13). Therefore, these procedures are now performed in a negative-pressure room (with an anteroom) using a positive air–purifying respirator suit.

The second physician (case-patient 11) may have become infected because the N95 mask was poorly fitting. Alternatively, transmission may have occurred through the conjunctival mucosa. All healthcare workers are now required to use eye protection when examining patients. Fit-testing of N95 masks is also mandatory, in addition to training on the correct use and disposal of PPE.

SARS has demonstrated remarkably efficient transmission in the hospital environment (76% of Singapore cases were nosocomially acquired) (3). At our hospital, nosocomial transmission occurred in nine persons. Our hospital structure of open wards with large numbers of beds separated by curtains may have been a contributory factor to the spread of the virus. These wards are subdivided into cubicles of four to eight beds and have open windows and ceiling fans with no controlled airflow patterns. Another likely factor was the failure to implement a policy of universal PPE use early in the outbreak. Where such measures were implemented (i.e., isolation rooms), no nosocomial transmission occurred.

In addition, because hospital staff are a recognized source of secondary transmission (3,4,6), all healthcare workers are now required to monitor their temperatures three times a day. Anyone with a respiratory illness or a temperature >37.5ºC is removed from duty, pending further evaluation. This policy has successfully prevented the secondary transmission of SARS from affected healthcare workers.

The lessons gathered from our hospital outbreak have resulted in dramatic changes to our triage and infection-control policies. All patients with undifferentiated febrile illness, respiratory complaints, or chest x-ray infiltrates are isolated, screened for SARS contacts, and nursed with full PPE. Despite continued community transmission of SARS in Singapore (the last community case was identified on May 10, 2003), the measures implemented since April 8, 2003, enabled us to identify and contain eight additional SARS cases and prevent the nosocomial transmission of the virus.

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West Nile Virus Transmission in Resident Birds, Dominican Republic

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We report West Nile virus (WNV) activity in the Dominican Republic for the first time. Specific anti-WNV antibodies were detected in 5 (15%) of 33 resident birds sampled at one location in November 2002. One seropositive bird was <4 months old, indicating a recent infection.

The initial outbreak of West Nile virus (WNV; family *Flaviviridae,* genus *Flavivirus*) in the Western Hemisphere took place in New York in 1999, with deaths observed in humans, horses, and numerous species of wild birds (1). Since then, this virus has spread rapidly across North America (2,3). Migratory birds are suspected of being responsible for the rapid spread of WNV through North America (4), and transport of WNV by Neotropical migratory birds throughout the New World has been anticipated (5).

Although WNV has spread rapidly through continental areas, its ability to spread across oceanic barriers is uncertain. The many islands of the West Indies represent the wintering grounds of numerous North American migratory birds (6,7) that breed in or migrate through WNV transmission foci in the United States. The Caribbean islands tend to have high human population density, and low populations of many birds and other vertebrates are restricted to certain islands. Introduction of WNV to the West Indies would present a human and equine health concern and potentially threaten numerous endangered and endemic bird species and perhaps other wild vertebrates.

Given the speculation that WNV may be disseminated by migrating birds (5,8,9), we hypothesized that the virus would be introduced to the Dominican Republic. Accordingly, we sampled apparently healthy birds there for evidence of locally acquired WNV infection.

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The Study

Birds were studied at two sites in the Dominican Republic, on the island of Hispaniola (Figure): Parque Nacional Sierra de Baoruco (November 7–16, 2002; 18° 12' N, 71° 32' W) and Parque Nacional Los Haitises (November 18–23, 2002; 19° 00' N, 69° 30' W). Birds were collected by standard methods (10). Tissues (eye, spleen, and kidney) were removed from 89 birds of 29 species (25 resident, 4 migratory) at Sierra de Baoruco and from 58 birds of 27 species (18 resident, 9 migratory) at Los Haitises; the tissues were tested for active WNV infection. Blood samples were collected from a subsample of these birds, including 41 that represented 18 resident species at Sierra de Baoruco and 33 that represented 16 resident species at Los Haitises. Blood was not collected from migratory birds or from certain very small resident birds, such as hummingbirds. Blood and tissue specimens were frozen immediately in liquid nitrogen for transportation and then stored at –70°C. Voucher specimens (including additional tissue samples) were prepared for all birds and are deposited at the University of Kansas Natural History Museum (KUNHM).

The sex and breeding condition of each bird were determined by examination and measurement of gonads. Birds' ages were assessed by plumage, skull ossification, and presence or absence of a bursa of Fabricius. The migratory or resident status of each bird was determined on the basis of standard references (6,7). For species that had both migratory and resident populations, we based status assessment on breeding conditions (breeding birds were assumed to be resident).

Serum samples were screened for flavivirus-neutralizing antibodies by plaque-reduction neutralization test (PRNT) according to standard methods (11) and by using challenge inocula of 100 plaque-forming units (PFU) WNV strain NY99-4132 and Saint Louis encephalitis virus (SLEV) strain TBH-28. Testing for neutralizing antibody

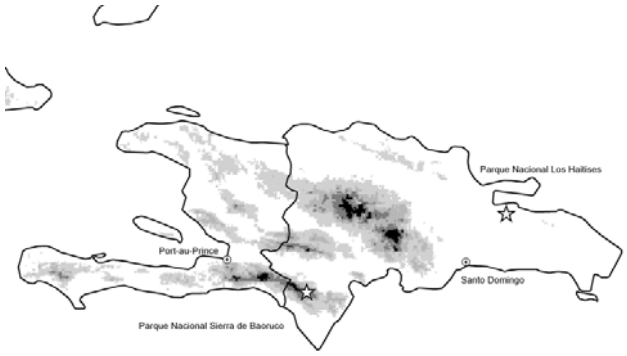

Figure. Hispaniola in the West Indies, on which are located Haiti (western third of the island) and the Dominican Republic. West Nile virus transmission occurred at Parque Nacional Los Haitises before November 2002. Shades of gray are 500-m intervals (e.g., 0–500, 500–1000).

to SLEV was important because this virus has been detected in the Caribbean and isolated from wild birds (12) and cross-reacted to anti–WNV-neutralizing antibodies in 6% of seropositive birds sampled in New York (13). PRNTs were performed with Vero cells in 6-well plates and a serum dilution of 1:10 in BA1 buffer (Hanks M-199 salts, 0.05 M Tris pH 7.6, 1% bovine serum albumin, 0.35 g/L sodium bicarbonate, 100 U/mL penicillin, 100 mg/L streptomycin, 1 mg/L Fungizone). Specimens that neutralized the virus stocks by at least 80% were further titrated in duplicate. To identify either virus as the causative agent, we used 90% neutralization as the criterion for a positive test result, and a four fold greater titer to one of the flaviviruses was considered diagnostic for that flavivirus.

Serum samples that showed neutralizing antibody titers to WNV or SLEV were tested by epitope-blocking enzyme-linked immunosorbent assays (ELISAs) with the WNV-specific monoclonal antibody 3.1112G, which discriminates between WNV and SLEV infections in birds (14). Because of the current lack of information on flaviviruses in the Dominican Republic, we required both the PRNT and the ELISA to test positive to consider a serum sample positive for WNV.

Tissues from each individual bird were pooled and homogenized in 2 mL of BA1 supplemented with 20% fetal bovine serum. Homogenates were clarified by centrifugation at 3,700 rpm for 10 min at 4°C. Four hundred microliters of each homogenate was screened for virus by Vero plaque assay (11). Homogenates obtained from flavivirus-seropositive birds were assayed for WNV RNA by TaqMan reverse-transcription polymerase chain reaction with WNV-specific primers (15).

Serum samples from nine resident birds tested positive for flavivirus-neutralizing antibodies (Table). Of these, five birds were positive for WNV antibodies by PRNT and blocking ELISA. All WNV antibody–positive birds were sampled at the Los Haitises study site. One serum sample was collected from an immature bird (ruddy quail-dove, *Geotrygon montana*; KUNHM 94667) that was <4 months old, suggesting that virus transmission was recent. Virus was not isolated from any of the tissues tested from 118 resident and 29 migratory birds (all migrants were Parulidae), nor did we detect WNV RNA in any of the tissue homogenates from flavivirus-seropositive birds.

Conclusions

Our finding of WNV-neutralizing antibodies in five resident birds represents the first evidence of WNV activity in the Dominican Republic. No cases of WNV infection in humans, horses, or birds were known at the time of sampling. The birds in this study could have been infected with WNV in the Dominican Republic as recently as early November 2002; nonetheless, the virus probably arrived earlier in the Caribbean region. Because no current infections were detected, our results reflect past virus transmission activity. Although we cannot determine when this activity began, the seropositive immature quail-dove presumably was infected after mid-July 2002, when it was

Table. Laboratory results for flavivirus-seropositive birds collected in the Dominican Republic, 2002[a]

Species	KUNHM catalog no.	Date sampled	Age and sex[b]	Locality	SLEV $PRNT_{90}$	WNV $PRNT_{90}$	% inhibition by ELISA[c]	Result
Ruddy quail-dove (*Geotrygon montana*)	94667	21 Nov	Immature male	Los Haitises	<10[d]	20[d]	40	WNV
Mangrove cuckoo (*Coccyzus minor*)	94671	19 Nov	Adult female[e]	Los Haitises	<10	160	81	WNV
Hispaniolan lizard cuckoo (*Saurothera longirostris*)	94669	18 Nov	Adult female	Los Haitises	160	640	73	WNV
Hispaniolan lizard cuckoo (*Saurothera longirostris*)	94670	21 Nov	Adult male	Los Haitises	<10	40	23	FLAV
Hispaniolan trogon (*Priotelus roseigaster*)	94951	12 Nov	Adult male	Sierra de Baoruco	<10	10	6	FLAV
Red-legged thrush (*Turdus plumbeus*)	94956	20 Nov	Adult female[e]	Los Haitises	10	160	86	WNV
Red-legged thrush (*Turdus plumbeus*)	94689	19 Nov	Adult male[e]	Los Haitises	<10	80	Not available	FLAV
Red-legged thrush (*Turdus plumbeus*)	94691	21 Nov	Adult female	Los Haitises	20	1280	61	WNV
Greater Antillean grackle (*Quiscalus niger*)	94949	19 Nov	Adult male	Los Haitises	640	40	42	FLAV[f]

[a]ELISA, enzyme-linked immunosorbent assay; FLAV, undifferentiated flavivirus; KUNHM, University of Kansas Natural History Museum, Division of Ornithology; $PRNT_{90}$, reciprocal 90% plaque reduction neutralization titer; SLEV, Saint Louis encephalitis virus; WNV, West Nile virus.
[b]Birds were in nonbreeding condition unless otherwise indicated.
[c]Inhibition values ≥30% were considered significant.
[d]Values represent reciprocal titers; threshold of detection was 1:10.
[e]Breeding condition, as determined by size of gonads.
[f]Although serologic results based upon PRNT would suggest that this specimen be identified as SLEV antibody–positive, the WNV antibody–positive result in the blocking ELISA indicates that this specimen was possibly positive for both SLEV and WNV. However, secondary flavivirus infections are notorious for heterologous reactivity, so infection by WNV or other flaviviruses causing these reactions could not be ruled out. Hence, the determination as FLAV.

born, and before early November 2002, in order to have stimulated detectable antibody production by mid-November.

The earliest evidence of WNV transmission in the West Indies is a human case from the Cayman Islands in 2001 (16). WNV-seropositive birds captured in Jamaica early in 2002 may have been infected in 2001 or earlier (17). Additional evidence of WNV transmission in the Caribbean region includes the report of two seropositive horses in Yucatán state, Mexico, sampled in July 2002 (18). Although the seropositivity of vertebrates in Cayman Islands, Jamaica, Mexico, and now the Dominican Republic is strong evidence for WNV activity in the region, it is indirect evidence and does not entirely rule out the possibility of cross-reactions with another flavivirus in laboratory assays. WNV remains to be isolated from the region.

The presence of WNV at Los Haitises may have resulted from transportation by viremic migratory birds from North America, where WNV transmission foci are widespread (2). Several migratory bird species, in particular, parulid warblers (order Passeriformes), were observed at this site. At least some passerine birds are capable of transmitting virus during their few days of viremia (19). Therefore, transmission to mosquitoes or predators from viremic migrants would be possible for a brief period (a few days, at most) after arrival at a site. Virus introduction into Caribbean ecosystems is therefore likely to occur at coastal sites where transoceanic migrants make first landfall.

We found no evidence of active virus in bird tissues of both resident and migratory species. We did not test serum samples from migrants because the presence of antibodies would not be informative, given the history of these birds traveling through areas of WNV transmission in or near North American breeding grounds. We presume that the five seropositive resident birds were infected locally because the four species involved are not migratory (7). Although young birds may disperse several kilometers from natal sites (20), adults probably live entirely within breeding territories.

Although only five birds (15%, 95% confidence interval [CI] 5% to 32%) at Los Haitises were found to be seropositive, the results suggest that transmission of WNV among bird populations at that site was widespread. For comparison, seroprevalence of WNV-neutralizing antibodies in resident birds was 50% (CI 44% to 57%) in Queens, New York City, after the 1999 outbreak (13), and 23% (CI 18% to 29%) in Staten Island after the 2000 outbreak (21). We used conservative criteria for determining a positive result because the background diversity of flaviviruses in the Dominican Republic has not been studied recently. If only the PRNT had been used (as was the case in the New York studies), then seven (21%, CI 7% to 35%) of the birds from Los Haitises would have been reported as positive for antibodies to WNV.

The evidence for local WNV transmission in the Dominican Republic indicates risk for West Nile fever and meningoencephalitis in the human, equine, and avian populations of Hispaniola. We suggest that WNV be considered in the differential diagnosis of humans and other vertebrates with central nervous system disease in Hispaniola.

Acknowledgments

We thank Bolivar Cabrera, Tristan Davis, Juan Cepedes Hidalgo, José Dolores Jiménez, Esteban López-Medrano, and Domingo Sirí for assistance in the field; José Ramón Albaine and Simón Guerrero for logistical assistance and assistance with permits; John Roehrig for assisting with the interpretation of serologic test results; and the two anonymous reviewers who provided comments on the manuscript.

This study was supported in part by National Science Foundation Grant #0211388, and in part by the Centers for Disease Control and Prevention contract U50/CCU 820510-02.

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West Nile Virus Encephalitis and Myocarditis in Wolf and Dog

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In the third season (2002) of the West Nile virus epidemic in the United States, two canids (wolf and dog) were diagnosed with West Nile virus encephalitis and myocarditis with similarities to known affected species (humans, horses, and birds). The West Nile virus infections were confirmed by immunohistochemistry and polymerase chain reaction.

Since its 1999 introduction in New York, West Nile virus (WNV) has spread to >40 states, causing seasonal mosquito-borne disease in humans, horses, and birds (1–7). We recently identified two Illinois canids (a captive wolf and a domestic dog) with severe disease associated with WNV infection.

Outside the Western Hemisphere, WNV has been endemic for decades (4,8–11). Canids have not been thought to be important in the epidemiology of this virus. However, a dog with neurologic disease that died in Africa in 1977 is now thought to have been infected with WNV (9,12). In a recent study in which four dogs were experimentally infected, no clinical disease was observed, and a low viremia developed in one dog (11). Natural infections occur in dogs, as indicated by serum antibodies; seropositivity in surveys was 37% in the 1980s in South Africa, 24% in the 1990s in India, and 5% in 1999 in New York City (10,11,13). In addition, a few individuals of some mammalian species have been listed as infected (not diseased): 14 bats, four rodents, three rabbits, two cats, two raccoons, and a skunk (6).

The Study

Two Illinois canids were brought in for necropsy in August 2002: 1) a 3-month-old female wolf pup, which died after 2 days of lethargy, depression, and irritability that progressed to anorexia, weakness, ataxia, and blindness and 2) an overweight 8-year-old castrated male Irish Setter-Golden Retriever mixed breed dog that was euthanized in moribund condition after a 7-day illness. The dog was hospitalized after 3 days of lethargy, anorexia, polydipsia, ocular discharge, and difficulty in rising that had progressed to fever, listlessness, weakness, ptyalism, nasal and ocular discharge, watery diarrhea, and abdominal pain. Although the diarrhea resolved and the dog was stronger and more alert the morning after hospitalization, over the next 3 days, multisystemic clinical signs developed, including dyspnea, diarrhea with melena, ataxia, a head tilt with head bobbing, pulmonary edema, and cardiac arrhythmias. While hospitalized, the dog had mild anemia without spherocytes, moderate to severe thrombocytopenia with large platelets, moderate leukocytosis with a left shift, a moderate hypokalemia, a mild hypoglycemia, and hypoproteinemia. Symptomatic and supportive care treatment included broad-spectrum antibiotics, a gastrointestinal protectant, a diuretic, fluid therapy, and systemic corticosteroids (for immune-mediated thrombocytopenia and anemia).

Both the wolf and dog were necropsied. Samples of multiple tissues were fixed and processed for routine diagnostic histopathology. In addition, tissues were screened with peroxidase immunohistochemistry for distemper, rabies, WNV, *Toxoplasma,* and *Neospora*. Sections for WNV immunohistochemistry were pretreated with 0.1% protease (20 min at 37°C) and nonspecific antibody binding blocked with Power Block (BioGenx, San Ramon, CA). The primary antibody was mouse anti-WNV monoclonal ascitic fluid diluted 1:1,000 (ATTC, Manassas, VA). The wolf brain was assayed for rabies antigen by fluorescent antibody (Public Health Laboratory, Springfield, IL).

West Nile viral RNA in the brain of the wolf and the kidney and liver of the dog (available cyropreserved tissue) was assayed by using a modified 5′ nuclease fluorogenic real-time, reverse transcriptase polymerase chain reaction assay (RT-PCR) (TaqMan, Applied Biosystems, Foster City, CA) (14). Each amplification reaction contained primers and probes (Table) (14,15) for both WNV and St. Louis encephalitis virus (SLEV) with FAM (6-carboxyfluorescein) and VIC (Applied Biosystems) fluorescent-labeled probes, respectively. A tissue sample (about 200 µg) was homogenized in 1.5 mL of medium 199 with L-glutamine, Hanks balanced salt solution, and 25 mM HEPES in a microfuge tube with three 3.2-mm stainless steel beads (Biospec Products, Bartlesville, OK). The sample was ground in a mixer mill for four min at a rate of 1/30 sec^{-1}, and then centrifuged (4,000 X *g,* 4 min). RNA was purified from 220 µL of the supernatant by using a viral RNA purification kit and the Biorobot 9604 (QIAGEN, Inc., Alameda, CA), eluting in a final volume of 85 µL. Real-time, RT-PCR was done in a 25-µL reaction with One-step RT-PCR Master Mix kit (Applied Biosystems) containing 25 pmol of each primer set, 3.25 pmol of each

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Table. Sequence (5´ to 3´) of primers and probes used in real-time reverse transcriptase–polymerase chain reaction to assay for West Nile virus genome in canid tissue

Item	West Nile virus	St. Louis encephalitis virus
Forward primer	CAGACCACGCTACGGCG	GAAAACTGGGTTCTGCGCA
Reverse primer	CTAGGGCCGCGTGGG	GTTGCTGCCTAGCATCCATCC
Probe	TCTGCGGAGAGTGCAGTCTGCGAT	TGGATATGCCCTAGTTGCGCTGGC

probe, and 10 µL of the RNA extract. The amplification reaction was run in an Applied Biosystems Sequencing Detection System 7000 programmed for 48.0°C for 30 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. RNA samples from WNV isolate NY99 (5) and SLEV were run in parallel for positive controls for the amplification.

At necropsy, the wolf had no gross lesions. Histologically, the brain had encephalitis compatible with a viral infection, including scattered blood vessels with narrow rims of lymphocytes. Scattered in the gray matter of all brain sections were random, poorly demarcated aggregates of microglial cells and lymphocytes with rare neutrophils and mild necrosis including an occasional necrotic neuron (Figure 1A). The white matter contained rare glial nodules. Each of two sections of heart had a small focus of myocardial cell fragmentation with a few lymphocytes and macrophages and fewer neutrophils. A similar smaller focus was in one of the two sections of skeletal muscle. The outer cortex of the adrenal gland had several foci of two to five necrotic cells with a few lymphocytes. No major histologic changes were evident in the kidney, lung, liver, spleen, tonsil, pancreas, small intestine, colon, sciatic nerve, or bone marrow.

The dog had several abnormalities at necropsy. The major gross lesion was fibrinous epicarditis of the atria.

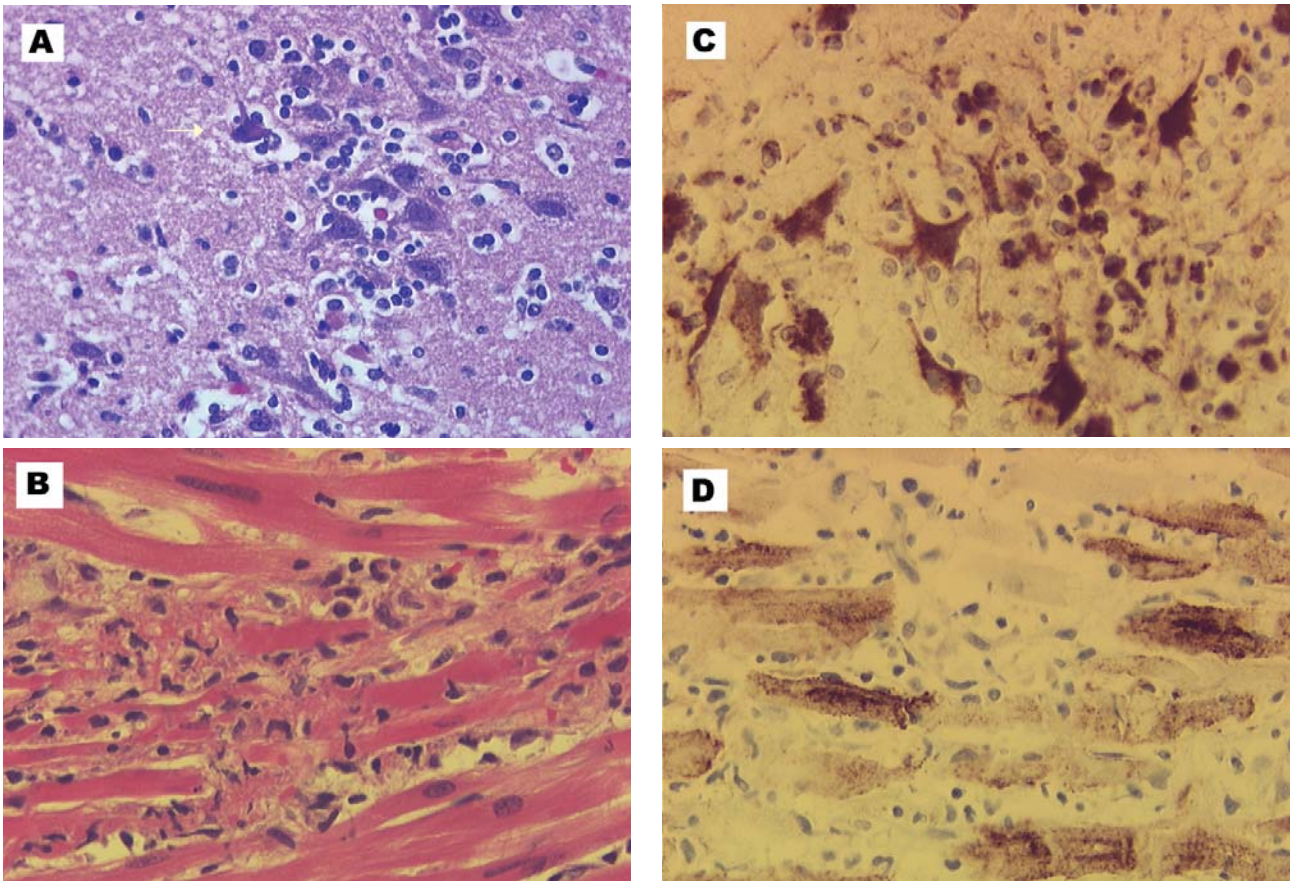

Figure 1. Encephalitis and myocarditis in two West Nile virus–infected canids. A, histopathology of the cerebrum of the wolf. Focus of lymphocyte infiltration and necrosis with mild gliosis; arrow indicates necrotic neuron. Hematoxylin-eosin staining. B, histopathology of the heart of the dog. Focus of leukocytic infiltration, mostly lymphocytes, and myocardial cell degeneration and necrosis. Hematoxylin-eosin staining. C, West Nile virus immunohistochemistry of the cerebrum of the wolf. Intense labeling of neurons in focus of inflammation. Some labeling is also in the glial cells or lymphocytes. Immunoperoxidase with hematoxylin counterstain. D, West Nile virus immunohistochemistry of the heart of dog. Intense labeling of most myocardial cells in the field. Immunoperoxidase with hematoxylin counterstain.

Other important findings included a hepatopathy (mottled tan yellow with an uneven surface), pulmonary edema, and two acute splenic infarcts. Histologically, the dog had a polioencephalitis similar to the wolf's, but much milder. In addition, the basal ganglia area had a focus of malacia predominately cleared via foamy macrophages. The dog had marked myocarditis (especially the atria) with numerous loose aggregates of leukocytes associated with degenerated or necrotic myocardial cells (Figure 1B). Lymphocytes predominated in the infiltrate; an occasional macrophage and neutrophil contributed. Both the lung and liver had changes compatible with heart failure: pulmonary edema, marked hepatic congestion, and marked hepatocellular fatty change with cholestasis. In addition to the acute infarcts, the spleen had marked lymphoid atrophy and marked hematopoiesis with hemosiderosis. The bone marrow was hyperplastic with proliferation of all three hematopoietic lineages. The hemogram, melena, and necropsy hematopoietic findings are most likely due to immune-mediated thrombocytopenia and anemia, a sporadic idiopathic condition in dogs.

No antigen was detected in either canid for canine distemper, rabies, toxoplasmosis, or neosporosis, diseases that can cause encephalitis or myocarditis. In contrast, WNV immunolabeling was intense in the brain of the wolf (Figure 1C). Abundant, intense labeling was associated with the areas of inflammation; focally, the labeling was grossly visible. Many neurons had intense labeling, and less dense labeling was associated with glial cells or lymphocytes. In the gray matter between inflammatory nodules, scattered individual neurons labeled intensely. Also immunolabeled in the wolf were a few myocardial cells and several small clusters of cells in the zona glomerulosa of the adrenal gland. No labeling was identified in the kidney and spleen. In the dog, the heart had intense immunolabeling in many myocardial cells (Figure 1D); the most extensive labeling was in the atria. The labeling in the dog brain was weak and inconclusive, and no label was found in the spleen. The dog had viral immunolabeling in scattered small foci of cells in the adrenal zona glomerulosa and renal tubular epithelial cells.

In the PCR assay, the WNV amplicon was abundant in the samples from the wolf brain (Figure 2), the dog kidney (not shown), and NY99 isolate (Figure 2). Samples from the dog liver failed to generate an amplicon signal. The SLEV primers, as expected, generated amplicons for SLEV control genome; the primers did not generate amplicons from samples of canid tissue (Figure 2).

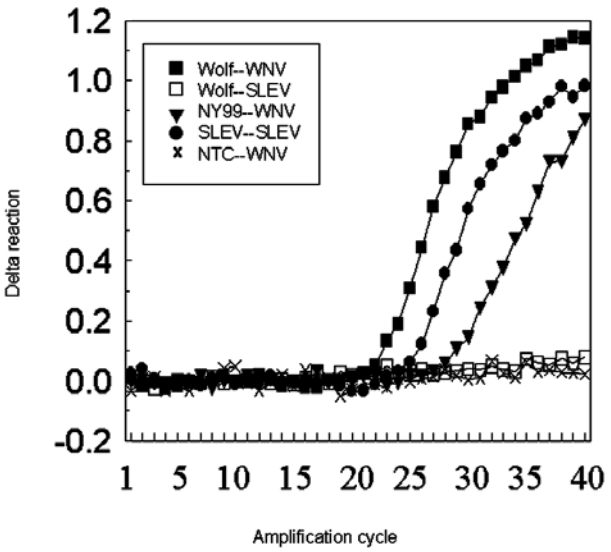

Figure 2. Real-time reverse transcriptase polymerase chain reaction of RNA extracted from the wolf brain. The amplification was duplexed with primers and probes for West Nile and St. Louis encephalitis viruses detected with fluorochrome dyes, FAM and VIC, respectively. Test and control samples were run in parallel and in duplicate (extraction and amplification) with consistent results. Delta reaction on the y axis represents the change in threshold fluorescence. The box lists the source of the template nucleic acid and the specificity of the fluorochrome probe (template source-probe specificity). WNV, West Nile virus; NY99, an isolate from the 1999 WNV outbreak in New York; SLEV, St. Louis encephalitis virus; NTC, no template control.

Discussion

The dog and wolf reported here are the first canids reported with WNV disease in the U.S. outbreak. The encephalitis in the canids affected the gray matter similar to WNV disease in horses, whereas humans have inflammatory nodules in both the gray and white matter (2,16,17). The myocarditis in the dog more closely resembled the lesions of WNV-infected birds than the lesions reported in mammals (2,3,17). Although crows and blue jays usually die without evidence of inflammation, WNV antigen is frequently demonstrated by immunohistochemistry in the hearts and in infected raptors, myocarditis is a common finding (J. J. Andrews, pers. comm.). The dog apparently had a concurrent, immune-mediated disease, which may have increased susceptibility for WNV disease.

Both of the canids likely were infected by bites from infected mosquitoes. WNV has been detected in 12 species of mosquitoes in Illinois, and four of these species prefer mammalian hosts (R. Novak, unpub. data). Novel routes of infection are also possible, such as ingestion of infected birds. The epidemiology of WNV in canids is likely similar to that in humans: sporadic disease cases with no important role in viral transmission or maintenance. More investigation is needed to confirm the epidemiology of WNV in canids and to monitor disease in other known and novel host species.

Acknowledgments

We thank Marshall Van de Wyngaerde and Nina Krasavin for the polymerase chain reaction assays, Jane Chladny and her co-workers for the histology preparation and immunohistochemistry assays, Chris Johnson and Hope Lopez for clinical care data on the two canine patients, and Laura Kramer for providing St. Louis encephalitis virus.

The work was supported in part by Centers for Disease Control and Prevention grant U50/CCU520518-01 (RJN) and Department of Natural Resources Illinois Waste Tire Fund (RJN).

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Weissella confusa Infection in Primate (*Cercopithecus mona*)

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We describe the first systemic infection by *Weissella confusa* in a mona monkey (*Cercopithecus mona*) on the basis of microbiologic, molecular genetic, and histologic data. The same strain of *W. confusa*, as determined by pulsed-field gel electrophoresis, was isolated in pure culture from the brain, liver, spleen, and intestine of this primate, illustrating the clinical importance of the isolations.

Weissella microorganisms are gram-positive, catalase-negative coccobacilli, which have been isolated from a wide variety of habitats such as soil, fresh vegetables, fermented foods, or meat and meat products (1,2). The genus *Weissella* is peculiar since it currently includes 11 validated species, but only *Weissella confusa* (basonym *Lactobacillus confusus*) and *W. cibaria* have been isolated from human or animal clinical sources. *W. cibaria* has been isolated from human bile and feces, the liver of a canary, and material from an infected ear in a dog (1). *W. confusa* has been isolated from feces of children with bacteremic infections (3) and liver transplants (4), and from the peritoneal fluids and abdominal walls of two patients (5). In animals, *W. confusa* has been isolated from necropsy specimens from a dog and from the ear of a dog with otitis (1). However, with the exception of a thumb abscess caused by *W. confusa* in a healthy 49-year-old man (6), the clinical significance of all other clinical isolates was not clearly established. This article describes the first well-documented systemic infection caused by *W. confusa* in a primate.

Case Report

A juvenile female mona monkey (*Cercopithecus mona*) was found dead without clinical signs of disease in the previous 24 h. The animal had no previous relevant medical history. The monkey was housed in a cage with another monkey, which formed part of a primate bioacoustic research unit. None of the other monkeys housed in the same research unit died or exhibited any clinical sign. The dead monkey was sent to the hospital of the veterinary school at the Complutense University in Madrid for necropsy. Postmortem examination showed the existence of congestion, edema, and petechial hemorrhages in most internal organs, especially marked in the brain, liver, and spleen, which are typical lesions associated with systemic infections. Samples from intestine, lung, liver, and brain were collected under aseptic conditions for microbiologic studies. Tissue samples were surface-plated on Columbia blood agar (bio-Mérieux España, s.a. Madrid) and incubated aerobically and under anaerobic conditions for 48 h at 37°C. Gram-positive, catalase-negative, facultative anaerobic coccobacilli were isolated in pure culture from lung, liver, brain, and intestine. Biochemical characterization was achieved by using the commercial Rapid ID32 Strep version 2.0 system (bioMérieux España, s.a. Madrid) according to the manufacturer's instructions. The four isolates had identical biochemical profile (numerical code 72007000000), being identified as *Leuconostoc* spp. Acid production from ribose, L-arabinose, and galactose was also tested by using phenol red base medium (Difco Laboratories, Detroit, MI), supplemented with 1% (w/v) sugar, after 48 h of incubation at 37°C. Antimicrobial susceptibility was tested by the microdilution method and haemophilus test medium with lysed horse blood (7) with a commercially prepared dehydrated panel (Sensititre**,** Trek Diagnostic System, East Grinstead, UK) as previously described (8). MICs (in μg/mL) were as follows: tetracycline, ≤1; amoxicillin, ≤0.25; trimethoprim, 32; erythromycin, ≤0.5; penicillin, ≤0.5; chloramphenicol, 8; ciprofloxacin, ≤0.25; and vancomycin ≥128. Resistance of *W. confusa* to vancomycin has been reported previously (4,6,7).

For histopathologic studies, tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, cut in 4-μm sections, and stained with hematoxylin and eosin. Histologic examination of the lungs, spleen, and liver showed the existence of inflammatory infiltrates composed mainly of neutrophils, and in lower proportion, of lymphocytes and macrophages (Figure 1), suggesting the existence of an acute septicemic process. Gram-positive coccobacilli emboli were observed in some hepatic vessels, suggesting a hematogenous dissemination.

Identifying *Weissella* species by classic phenotypic methods can be difficult (1,9). Comparing the 16S rRNA gene sequences of bacterial species is a useful approach for the identifying unusual clinical isolates or those which cannot be properly identified by conventional phenotypic methods (10,11). The 16S rRNA gene of each isolate was amplified by polymerase chain reaction (PCR) and further sequenced to determine genotypic identity (12). The determined sequences consisted of approximately 1,400

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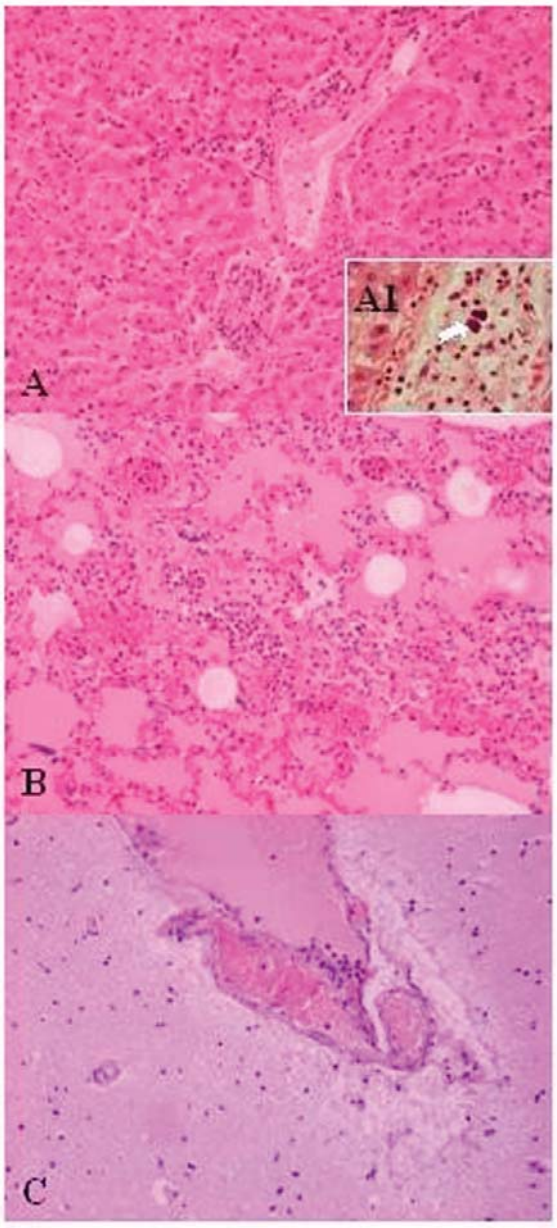

Figure 1. Lesions in the mona monkey (hematoxylin and eosin stain): A) liver: portal triads with neutrophilic infiltration (x10); A1, presence of bacterial emboli inside the vein (arrow) (x40). B) acute pneumonia: edema, congestion, and leukocyte cells exudation in the pulmonary alveoli (x10). C) encephalitis: congestion and marginalized neutrophils in nervous vessels (x10).

nucleotides and were compared with the sequences of other gram-positive, catalase-negative species available in the GenBank database, by using the BLAST program (available from: URL: http://www.ncbi.nlm.nih.gov/BLAST). The 16S rRNA gene analysis indicated that the four isolates were genotypically identical, displaying the closest sequence similarity (99.9%) with *W. confusa* (accession no. AB023241). Sequence similarity with *W. cibaria* was 99.2%, which agrees with the high sequence similarity reported for both species (1). Overall results of the phenotypic characterization of the clinical isolates were consistent with those described for this species (13). Like *W. confusa*, clinical isolates were able to produce acid from ribose and galactose but not from L-arabinose, one of the few biochemical tests that can differentiate this species and *W. cibaria* (1). These results support the identification of the clinical isolates as *W. confusa*. *Weissella* microorganisms can be isolated as normal flora of the intestinal tract (l,14,15). Thus, an extraintestinal origin of the systemic infection is most likely.

W. confusa isolates were molecularly characterized by pulsed-field gel electrophoresis (PFGE), according to the specifications of Vela et al. (16) with the CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA). The restriction enzymes *Apa*I (Promega UK Ltd., Southhampton, UK) and *Sma*I (MBI Fermentas Vilinius, Lithuania) were used according to the manufacturer's recommendations. These enzymes have been successfully used for the molecular typing of microorganisms closely related to *Weisella* (17). Gels were interpreted by standard criteria (18). All *W. confusa* isolates displayed undistinguishable macrorestriction patterns by PFGE with *Sma*I (data not shown) and

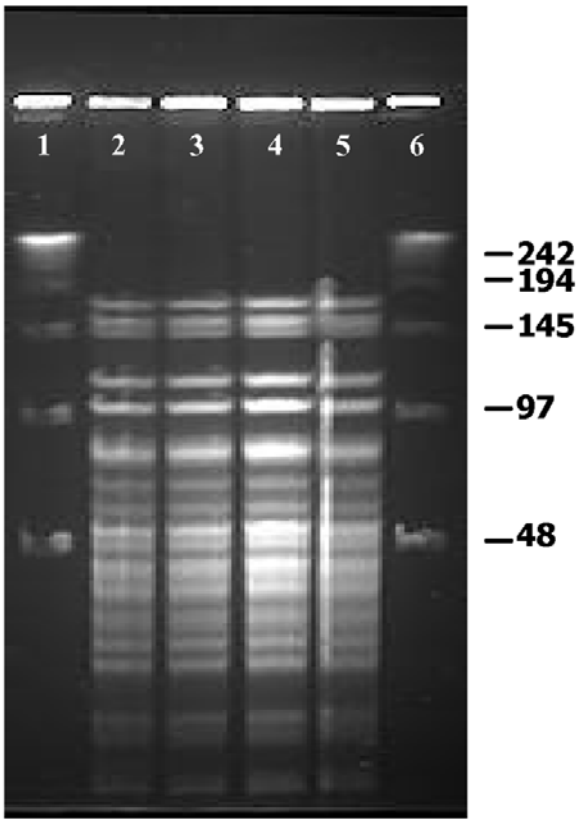

Figure 2. Pulsed-field gel electrophoresis profiles of *Apa*I-digested genomic DNA of *Weissella confusa* clinical isolates. Lanes 1–4: Isolates from intestine, brain, spleen, and liver, respectively.

*Apa*I (Figure 2) restriction enzymes, demonstrating that the systemic infection was caused by a single strain of *W. confusa*.

Conclusions

Weissella are considered nonpathogenic microorganisms because most of the strains isolated from clinical samples have been obtained as mixed cultures without evidence of their clinical significance (1,7). In this study, the same strain of *W. confusa*, as determined by PFGE, was isolated in pure culture from the brain, liver, and spleen; the isolations from these organs, together with the histopathologic data, illustrate the clinical importance of the isolations. These results generate further speculation about the potential of *W. confusa* as an opportunistic pathogen. This is the first well-documented study in which, by combining microbiologic, molecular genetic, and histologic, data, the clinical importance of the isolation of *W. confusa* and its implication in an animal infection is clearly established.

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Mycobacterium tuberculosis Beijing Genotype, the Netherlands

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To determine whether the Beijing genotype of *Mycobacterium tuberculosis* is emerging in the Netherlands, we collected data on 6,829 patients during 1993 to 2000. Six percent had the Beijing genotype. This genotype was associated with diagnosis in recent years, young age, nationality, and multidrug resistance.

The Beijing genotype is found frequently in Asia (1–3) but also in outbreaks of multidrug-resistant tuberculosis (MDRTB) in various parts of the world, including Cuba, Germany, Russia, and Estonia (4–7). The largest known epidemic of MDRTB in North America was caused by the W strain, a variant of the Beijing genotype (8–9). A recent study showed this strain's emergence and association with drug resistance in Vietnam (10). The Beijing genotype was also responsible for a recent outbreak of tuberculosis (TB) on the Canary Islands (11).

The relatively high degree of genetic conservation of Beijing genotype strains found in a widespread area (12) suggests a recent dissemination and, hence, selective advantages associated with this genotype of *Mycobacterium tuberculosis*. Recently, Beijing genotype bacteria were reported to carry mutations in putative mutator genes, which may explain a higher adaptability of these bacteria under stress conditions such as exposure to the intracellular environment or anti-TB drugs (13). These mutations may also be the basis of differential interaction between Beijing genotype bacteria and the host immune defense system suggested in a recent study in Indonesia (14).

In the Netherlands, MDRTB is affecting <1% of new TB patients, in particular immigrants (15–17). The incidence of the Beijing genotype has not yet been described. The goal of our study was to determine whether the Beijing genotype is emerging in the Netherlands and whether this genotype is associated with multidrug resistance.

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The Study

Patient data were obtained from the Netherlands Tuberculosis Register, which is maintained by the Royal Netherlands Tuberculosis Association and has been in place since 1993. Municipal health services, which are responsible for the followup of all TB patients, send information using standardized, precoded forms on all reported TB cases to the register. The register includes information on demographic characteristics, case detection, risk groups, type of disease, treatment, and treatment outcome.

Isolates obtained from all 8,210 culture-positive patients underwent restriction fragment length polymorphism (RFLP)–typing with IS*6110* as a probe at the National Institute of Public Health and the Environment. The Beijing genotype was defined on the basis of spoligotype (no spacers 1–34; at least 4 of spacers 35–43) and a specific region A insertion (18). Certain IS*6110* RFLP-genotype families (clades 47 and 61) were consistently found to be Beijing genotype (18). Isolates were assigned to the Beijing genotype on the basis of the IS*6110* RFLP pattern (18). If any doubt about clade membership existed, we used spoligotyping for the final allocation.

The RFLP results for the period 1993–2000 were matched to patient data on the basis of date of birth, postal area code, and sex, resulting in a perfect match for 5,994 (73%) patients and a near-perfect match for 835 (10%) patients; overall, 83% of the samples matched (6,829 patients). During this period, the Beijing genotype was observed in 516 cultures, representing 6% of the 8,210 patients. In the matched dataset, 415 (6%) of 6,829 were of the Beijing genotype. The number of TB cases attributable to the Beijing genotype tended to increase over time (Figure; $p>0.05$). This tendency was observed both among Dutch patients ($r=0.66$, 95% confidence interval [CI] –0.08 to 0.93) and non-Dutch patients ($r=0.59$, 95% CI –0.20 to 0.91) (Figure). While the increase in the number of cases among immigrants may be associated with an overall increase of immigrants to the Netherlands during

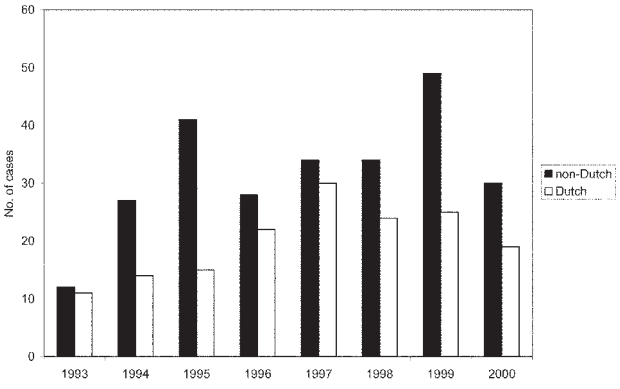

Figure. Number of tuberculosis cases with the Beijing genotype, Netherlands, 1993–2000.

Table. Risk factors for the Beijing genotype of *Mycobacterium tuberculosis*, the Netherlands, 1993–2000

Risk factor	Beijing genotype	Total	%	Odds ratio	
				Crude	Adjusted[b] (95% confidence intervals)
Y of diagnosis					
1993	23	669	3	1.09	1.08 (1.03 to 1.13)
1994	41	941	4		(per year)
1995	56	863	6		
1996	50	946	5		
1997	64	872	7		
1998	58	804	7		
1999	74	879	8		
2000	49	855	6		
Sex					
Male	254	4,135	6	1	
Female	161	2,694	6	0.97	
Age group					
<25	120	1,522	8	1	1
25–34	123	1,925	6	0.80	0.69 (0.53 to 0.91)
35–44	64	1,080	6	0.74	0.60 (0.43 to 0.83)
45–54	23	614	4	0.45	0.36 (0.23 to 0.57)
55–64	24	493	5	0.60	0.55 (0.35 to 0.88)
65–74	40	517	8	0.98	0.78 (0.52 to 1.15)
75+	21	678	3	0.37	0.30 (0.18 to 0.50)
Nationality					
Netherlands	160	2,825	6	1	1
Europe (central and eastern)	20	231	9	1.58	1.27 (0.77 to 2.09)
Turkey	3	339	1	0.15	0.12 (0.04 to 0.38)
Morocco	19	650	3	0.50	0.41 (0.25 to 0.68)
Somalia	21	957	2	0.37	0.27 (0.17 to 0.44)
Africa (other)	28	594	5	0.82	0.63 (0.41 to 0.96)
Asia	140	786	18	3.61	3.01 (2.32 to 3.91)
Other	19	345	6	0.97	0.86 (0.53 to 1.42)
Unknown	5	102	5	0.86	0.73 (0.29 to 1.84)
RFLP clustering[a]					
No	212	3,227	7	1	
Yes, first case	57	1,052	5	0.81	
Yes, later case	146	2,550	6	0.86	
Localization					
Pulmonary	268	4,064	7	1	
Extrapulmonary	113	2,122	5	0.80	
Pulmonary and extrapulmonary	34	643	5	0.79	
Residing in Netherlands					
<6 months	46	641	7	1	
6–11 mo	12	301	4	0.54	
12–23 mo	28	358	8	1.10	
2–4 y	53	767	7	0.96	
≥5 y	122	1,710	7	0.99	
Born in Netherlands	117	2,437	5	0.65	
No information	37	615	6	0.83	
Drug resistance					
Susceptible	332	5,910	6	1	
H only	11	227	5	0.86	
S only	34	358	9	1.76	
Other patterns	26	221	12	2.24	
Multidrug-resistant	9	53	17	3.44	
Unknown	3	60	5	0.88	
Multidrug resistance					
Yes	9	53	17	3.21	2.64 (1.22 to 5.74)
No	406	6,776	6	1	1
HIV infection					
Yes	14	294	5	0.76	
No	401	6,535	6	1	

[a]RFLP, restriction fragment length polymorphism.
[b]Adjusted for year of diagnosis, age, nationality, and multidrug resistance.

the study period, the increasing numbers among Dutch patients likely reflect an increasing incidence rate.

The proportion of TB patients who had the Beijing genotype was significantly associated in univariate analysis with a later year of diagnosis, young age, nationality (increased among immigrants from Asia and decreased among those from Morocco, Turkey, Somalia, and other African countries, compared with Dutch citizens), and multidrug resistance (Table). These associations persisted in multivariate analysis (Table).

The association between the proportion of TB cases with the Beijing genotype and age was observed in particular in Dutch patients (chi square$_{trend}$ 15.5; $p<0.0001$), and was not clear among non-Dutch patients (chi square$_{trend}$ 0.8; $p>0.2$). Among Dutch patients, the Beijing genotype was more commonly found in new cases (6%, 133/2,130) than among retreated cases (4%, 18/498; $p<0.05$). Among non-Dutch patients, the Beijing genotype was slightly more common in retreated cases (8%, 19/229) than in new cases (6%, 204/3,218); however, this occurrence was not significant ($p>0.2$). The incidence pattern by person and time appears consistent with transmission of the Beijing genotype from immigrants to the Dutch population (19).

The proportion of TB cases with the Beijing genotype was not substantially associated with HIV infection, pulmonary localization, RFLP clustering, or duration of stay in the Netherlands (Table). The lack of association with RFLP-clustering suggests that the Beijing genotype is not spreading more quickly than other *M. tuberculosis* strains.

Of the nine patients with the Beijing genotype and multidrug resistance, four were from central and eastern Europe, three from Asia, one from Africa, and one from the Netherlands. One of these nine patients was RFLP-clustered; the other patient in that RFLP cluster had been diagnosed previously and had isolated isoniazid resistance.

During 1993 to 2000 in the Netherlands, the Beijing genotype represented 6% of TB cases. The Beijing genotype was associated with recent diagnoses, young age (in particular among Dutch citizens), nationality (Eastern Europe and Asia), and multidrug resistance. Although the Beijing genotype was associated with multidrug resistance, the number of MDRTB cases was small. No secondary cases were observed with the Beijing genotype and MDRTB. Of the nine cases of MDRTB, only one case may have been caused by transmission within the Netherlands from a case-patient who had isoniazid resistance at the start of treatment and may have acquired rifampicin resistance during treatment. However, the spread of the Beijing genotype among young people suggests the possible emergence of multidrug resistance and emphasizes the need for continued surveillance.

Acknowledgments

We thank all municipal health services in the Netherlands for their contribution to the Netherlands Tuberculosis Register.

This study was performed within the framework of Concerted Action project QLK2-2000-00630 on Molecular Epidemiology of Tuberculosis.

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Saliva and Meningococcal Transmission

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Neisseria meningitidis carriage was compared in swab specimens of nasopharynx, tonsils, and saliva taken from 258 students. We found a higher yield in nasopharyngeal than in tonsillar swabs (32% vs. 19%, p<0.001). Low prevalence of carriage in saliva swabs (one swab [0.4%]) suggests that low levels of salivary contact are unlikely to transmit meningococci.

Invasive meningococcal disease has a high case-fatality rate and an immediate risk of further cases among household contacts. Public health measures therefore include prompt identification of contacts for chemoprophylaxis (1). One question that commonly arises is whether salivary contact through sharing cups or glasses is an indication for prophylaxis, but the evidence base to inform an answer is weak, and national guidelines are inconsistent (1,2). Although saliva is thought to inhibit meningococcal growth (3), carriage rates in saliva are not known, and swabs to detect carriage are usually taken from tonsils or nasopharynx (4–6). We compared meningococcal isolation rates in swabs of saliva (front of mouth), tonsils, and nasopharynx.

We recruited volunteers among students from two colleges in Hereford, England. After giving written consent, students completed a short questionnaire on age, sex, smoking, recent antimicrobial drug use, and meningococcal vaccine status. Three sterile, dry, cotton-tipped swabs were used to take samples from each volunteer: one from the nasopharynx (through the mouth and swept up behind the uvula), one from both tonsils, and one swab of saliva between the lower gum and lips. Swabs were plated directly onto a selective culture medium primarily designed for the isolation of pathogenic *Neisseria* species (modified New York City base containing vancomycin, colistin, and trimethoprim), prepared by Taunton Media Services, UK (7). The plates were transported to Hereford Public Health Laboratory, where they were spread once from the primary inoculum and incubated in 7% CO_2 at 37°C for 48 h. Putative *Neisseria* species isolated were sent to the Meningococcal Reference Unit, Manchester Public Health Laboratory, for *Neisseria meningitidis* confirmation and serologic phenotypic characterization. Data were entered into the computer using Excel (Microsoft Corp., Redmond, WA). Carriage rates by site were compared with McNemar's test and by risk factor using chi-square tests. Ethical approval was obtained from the Public Health Laboratory Service Ethics Committee and Herefordshire District Ethics Committee.

Of the 258 participants, 90 (35%) were identified as carrying *Neisseria meningitidis* from one or more sites. The site with the highest yield was the nasopharynx (32.2%), whereas tonsillar carriage was 19.4% (Table). One (0.4%) of the 258 saliva swab specimens was positive. No one had positive specimens from all three sites, and the person with the positive saliva swab had negative swabs from the other two sites. Differences in carriage rates between the nasopharynx and tonsils and between the nasopharynx and saliva were statistically significant (p<0.001 in both cases).

The predominant serogroup among carried strains was B. No serogroup C strains were identified. Of the 44 carriers with positive swabs from both nasopharynx and tonsils, each pair of isolates was considered to be phenotypically indistinguishable by serogroup, serotype, and sero-subtype. In three of these pairs, one isolate expressed serogroup B, and the paired isolate could not be serogrouped but had identical serotype and sero-subtype.

Of the 258 participants, 116 (45%) were men, and 142 were women. Most (86%) were 18 to 21 years of age. Carriage rates were higher among men than women (54/116 vs. 36/142, p<0.001), and among smokers than nonsmokers (49/90 vs. 51/168, p<0.001). Carriage rates were similar when persons were stratified by age, meningococcal vaccination status, and recent antimicrobial drug use. Although duplicate swabs from the nasopharynx sometimes yield different meningococcal strains (3), none of the paired isolates in this study were distinguishable by phenotype.

The yield of meningococci from nasopharyngeal swabs was nearly twice as high as that from tonsillar swabs. Previous researchers have found a lower sensitivity of nasopharyngeal swabs taken through the nose using small cotton-tipped wire swabs compared to tonsillar swabs taken using larger cotton-tipped swabs (5,6). Our use of the same type of swab to sample both sites provided a more valid comparison. The carriage rate was higher than expected for this age group (4), suggesting that we had

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[1]H.O. was responsible for recruiting students, obtaining specimens, swabs, and drafting the paper with J.S.; S.G. and M.M. were responsible for microbiologic processing and analysis; and J.S. designed the study and drafted the paper with H.O. All authors contributed to the final draft.

Table. Carriage of *Neisseria meningitidis* by site

Site of swab	One site positive	Two sites positive	Three sites positive	Total positive	Overall carriage %
Nasopharynx	39	44	0	83	32.2
Tonsils	6	44	0	50	19.4
Saliva	1	0	0	1	0.4

[a](n=258).

efficient swabbing and microbiologic techniques. We suggest that throat swabs to detect meningococcal carriage should always be taken from the nasopharynx (through the mouth whenever practical) and not from the tonsils.

The very low isolation rate from saliva swabs suggests that low levels of salivary contact are unlikely to transmit meningococci (1). This observation is supported by results of a case-control study among university students that found no association between meningococcal acquisition and sharing of glasses or cigarettes (8). On the basis of this evidence, we propose that guidelines for public health management of meningococcal disease should not include low-level salivary contact (e.g., sharing drinks) with a case-patient as an indication for chemoprophylaxis.

Acknowledgments

We thank all staff and students at Hereford College of Technology and Hereford College of Art and Design who were involved in this study and Erika Duffell and Nicky Maxwell for their help with sampling.

This study was supported by Gloucester Public Health Laboratory Trust Fund.

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Small Colony Variants of *Staphylococcus aureus* and Pacemaker-related Infection

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We describe the first known case of a device-related bloodstream infection caused by *Staphylococcus aureus* small colony variants. Recurrent pacemaker-related bloodstream infection within a 7-month period illustrates the poor clinical and microbiologic response to prolonged antimicrobial therapy in a patient infected with this *S. aureus* subpopulation.

Infections caused by *Staphylococcus aureus* range from mild skin infections to acute life-threatening diseases such as pneumonia, osteomyelitis, and endocarditis. However, *S. aureus* may also cause a chronic disease with persistent and recurrent infections. Skin and soft tissue infections, chronic osteomyelitis, and persistent infections in patients with cystic fibrosis have been associated with small colony variants, a naturally occurring subpopulation of the species *S. aureus* (1–6). *S. aureus* small colony variants are characterized as electron transport deficient bacteria because of their auxotrophism to hemin or menadione or are recognized as thymidine-dependent. These variants produce very small, mostly nonpigmented and nonhemolytic colonies. In addition, they also demonstrate various other features that are atypical for *S. aureus,* including reduced coagulase production, failure to use mannitol, and increased resistance to aminoglycosides and cell-wall active antibiotics (3–10). Furthermore, the ability of these variants to persist intracellularly within nonprofessional phagocytes has been described (3,5,11). Because of their fastidious growth characteristics and unusual morphologic appearance, small colony variants present a challenge both to the microbiologist and the clinician, often resulting in misidentification and misinterpretation (1,2,7,8). Prerequisite for recovering and identifying these variants is the application of extended conventional culture and identification techniques (3,5,8). We report the first case of a pacemaker-related bloodstream infection caused by *S. aureus* small colony variants. This case illustrates the poor clinical and microbiologic response to prolonged antimicrobial therapy in patients infected with these variants.

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Case Report

A 63-year-old man was transferred to our facility with the presumptive diagnosis of endocarditis related to a pacemaker-lead infection. Past medical history included hypertension, coronary artery disease, and noninsulin-dependent diabetes mellitus. A VVI (ventricular ventricular inhibited) pacemaker had been implanted for treatment of sick sinus syndrome 9 years earlier. Six weeks before admission, this device had been removed because of a pocket infection after blunt trauma with dislocation of the device and perforation of the skin. Specimens for microbiologic culture were not obtained at this time. The pacemaker leads were left in place, a gentamicin-containing sponge was applied to the infection site, and a new pacemaker was implanted on the other side of the chest. Four weeks later, the patient sought treatment at the local hospital for a high fever (39.7°C) and chills and a subcutaneous abscess with oxacillin-susceptible *S. aureus* at the primary insertion site. After surgical drainage, antimicrobial therapy was initiated with intravenous cefuroxime. The remaining pacemaker leads were partially cut but not completely removed. Ten days later, spiking fever and chills unresponsive to the administration of meropenem and vancomycin developed, and the patient was transferred to our medical center for pacemaker ablation. The physical examination did not indicate auscultation abnormalities or stigmata of endocarditis. Laboratory studies were unremarkable except a C-reactive protein (CRP) level of 170 mg/L (normal value $\leq$8 mg/L) and a blood sedimentation rate of 79 mm/h. Multiple blood cultures taken on admission remained negative. Transesophageal echocardiography did not show vegetations or other evidence of endocarditis. On hospital day 6, the new pacemaker was completely removed by percutaneous ablation as were the remaining leads of the old device. Only the tip of the pacemaker lead remained fixed in the myocardium, and surgical removal involving extracorporal circulation was not attempted. The patient's condition improved rapidly, CRP level returned to normal, and on hospital day 32, the patient was transferred to the local hospital to complete a 6-week course of intravenous vancomycin and rifampin as empirical antistaphylococcal therapy. Before transfer, the daily vancomycin dose had been reduced to 250 mg twice a day after an elevated vancomycin serum level. Eight days later, the patient was readmitted with recurrent high fever. Blood cultures taken on readmission were again negative. After the vancomycin dose was increased to 500 mg every 12 hours, the patient promptly became afebrile. Antimicrobial therapy

was discontinued after the patient had completed a 10-week course of vancomycin and rifampin. Three days later, the patient again had spiking fever. After 6 to 48 hours of incubation, four sets of blood cultures obtained on four consecutive days yielded nonpigmented and nonhemolytic staphylococci, initially identified on the basis of a negative tube coagulation test and the API ID 32 Staph system (bioMérieux, Marcy-L'Etoile, France) as coagulase-negative staphylococci, susceptible to oxacillin (MIC 0.5 mg/mL) and vancomycin (MIC 1.0 mg/mL) but resistant to rifampin (MIC >32 mg/mL). However, the colony morphologic findings were suggestive of small colony variants of *S. aureus*, confirmed by polymerase chain reaction amplification of the *nuc* and *coa* genes as well as by determination of the strain's auxotrophy for hemin. The patient responded promptly to flucloxacillin, 4 g intravenously three times a day. After another 6-week course of parenteral therapy, antimicrobial therapy was discontinued, and the patient was discharged (CRP 7 mg/L, ESR 35 mm/h); he was readmitted after 6 days with chills and high fever. Antimicrobial therapy with intravenous flucloxacillin was resumed and followed by immediate defervescence. Three blood cultures taken on readmission were again positive with *S. aureus* small colony variants. Clonal identity of all isolates was demonstrated by pulsed-field gel electrophoresis of bacterial DNA (data not shown). A transesophageal echocardiogram showed the residual tip of the pacemaker lead fixed in the myocardial septum without vegetations. The remaining device was finally removed by open-heart surgery with use of cardiopulmonary bypass. Microbiologic culture of the pacemaker electrode performed at a different institution yielded abundant growth of staphylococci that were misidentified as *S. warneri,* showing the same biochemical profile as the previously isolated bacteria as determined by the ID 32 Staph system. The patient recovered completely and was discharged on the 10th postoperative day after a total hospital course of 7 months.

Conclusions

S. aureus small colony variants have been implicated in persistent and recurrent infections that give a poor clinical and bacteriologic response to standard antimicrobial therapy in patients with abscess, chronic osteomyelitis, and bronchopulmonary infections, particularly after prolonged exposure to antibiotics (1–6). Bloodstream infection related to an implantable intravascular device with this *S. aureus* variant has not been reported before. These phenotypic variants are characterized by their fastidious growth and atypical colony morphologic findings on routine media, making recovery as well as correct identification difficult for microbiologic laboratories (3,5,8). The ability to interrupt electron transport and to form a variant subpopulation affords *S. aureus* a number of survival advantages, including the ability of this subpopulation to persist intracellularly within nonprofessional phagocytes (11,12). The intracellular position may shield small colony variants from host defenses and decrease exposure to antibiotics (3,5,11). *S. aureus* small colony variants can be selected by gentamicin in vitro and in vivo as shown in patients with osteomyelitis after gentamicin bead placement (4,12). Chuard et al. demonstrated that, in contrast to their normal phenotype parental strain, *S. aureus* small colony variants that were attached to fibronectin-coated coverslips were highly resistant to cell-wall–active antimicrobial agents such as oxacillin and vancomycin (13).

In our case, findings suggest that *S. aureus* small colony variants might have been selected from the parent strain population with a normal phenotype after exposure to the locally applied aminoglycoside or to the prolonged administration of vancomycin. Continually positive blood cultures with the same strain as demonstrated by molecular typing and the presumable persistence of these organisms on the pacemaker lead tip may partly be explained by the poor effectiveness of vancomycin and flucloxacillin against these slow-growing organisms that were adhering to the remaining foreign body and the ability of these variants to persist intracellularly (7,8,13).

This case adds to the spectrum of persistent and relapsing infections attributed to *S. aureus* small colony variants and emphasizes that these variants may also play a role in intravascular device–related infections. It also illustrates that complete removal of any foreign body material is essential for the complete cure of prosthetic intravascular device–related *S. aureus* infection. Laboratories should be particularly alert for *S. aureus* small colony variants when samples are submitted from patients who have received long-term antimicrobial therapy, especially if the infection is unusually persistent or recurrent.

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West Nile Virus Detection in American Crows

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A dipstick immunochromatographic assay used for West Nile virus (WNV) detection in mosquitoes was investigated for application to testing of fecal, saliva, and tissue samples from dead American Crows (*Corvus brachyrhynchos*). Results suggest that VecTest may be an efficient method for WNV detection in field-collected, dead American Crows, although confirmation of results and further investigation are warranted.

The American Crow (*Corvus brachyrhynchos)* has been designated as a West Nile virus (WNV) surveillance species (1), and dead American Crows have been used to monitor WNV activity across the nation. The American Crow is a useful species for monitoring disease activity because it is highly visible and recognizable; furthermore, all American Crows experimentally inoculated with WNV have died within 7 days of inoculation after attaining viremias of sufficient titer to infect mosquitoes (2). Avian deaths early in the transmission season are a warning for increased risk for human WNV cases (3); by monitoring WNV infection in dead American Crows, we can detect areas of epidemiologic public health concern.

Standard methods of identifying WNV in dead crows include two direct tests of tissues by immunohistochemistry (IHC) and reverse transcriptase-polymerase chain reaction (RT-PCR TaqMan (Applied Biosystems, Foster City, CA). Komar et al. (4) determined that postmortem cloacal and oral swabs could replace brain tissue as a specimen for WNV detection in crows and jays. Addressing this, and the need for a simple, quick, and cost-effective method for viral surveillance in dead crows, we conducted a study to determine whether the VecTest WNV/Saint Louis encephalitis virus (SLEV) Antigen Panel Assay (Medical Analysis Systems Inc., Camarillo, CA) could be used for WNV testing of fecal, saliva, and tissue samples from American Crows.

The VecTest was designed as a rapid wicking assay to identify the presence or absence of viral antigen specific to WNV or SLEV in infected mosquitoes. This test employs monoclonal antibodies against WNV and SLEV in a one-step procedure with a wicking test strip. All components necessary to carry out the test are provided in a kit, including vials, buffer solution, and dipsticks. The tests can be performed in the field, highly trained personnel and specialized equipment are not necessary, and results can be obtained quickly (<20 minutes). Each dipstick contains an internal positive control to indicate that the test has performed properly. After mosquitoes are ground in a buffer solution, a dipstick is inserted; WNV and SLEV antigen present in the mosquito slurry will bind to the specific antibody-colloidal gold conjugate. Antigen presence is indicated by a red line that develops in the test zone of the dipstick, specific to WNV or SLEV; pictures of dipsticks can be obtained from Ryan et al. (5). Ryan evaluated the product for detection of WNV in mosquitoes in a laboratory and suggested that sensitivity of VecTest is comparable to that of an antigen-capture enzyme-linked immunosorbent assay but less sensitive than Vero cell plaque assays or RT-PCR. Our study is a preliminary evaluation of the VecTest assay to determine whether it can be used for accurate WNV testing of field-collected dead American Crows.

The Study

In February–October 2002, as part of a broader study, we captured, banded, and marked 156 American Crows (*C. brachyrhynchos*) in Champaign and Urbana, Illinois (Yaremych SA. West Nile virus and American Crows in east-central Illinois. University of Illinois; 2003). Radiotransmitters were attached to individuals of a subsample of captured crows. Many of the study crows died in the summer; all recovered crows were tested for WNV. Dead crows were retrieved by tracking the radio signal to the carcasses, by chance encounter, or by notification from the public. Dead study crows with transmitters were typically recovered <36 hours after death, as this period was the maximum amount of time elapsed between the last live observation of a crow during radio tracking and recovery of the carcass upon death. We estimated that other marked study crows without transmitters were retrieved up to 72 hours after death. The abundance of dead crows allowed for a simple comparative study involving the use of VecTest, RT-PCR, and IHC tests to detect WNV in crow fecal, saliva, and tissue samples.

For each dead crow, we diluted fecal scrapings from the cloaca and salivary scrapings from the mouth in VecTest buffer solution in the vials provided in the kit. A metal spatula was used to obtain samples, and instruments were sanitized with a wash of soap and water and 70% ethanol before and after use. In some cases, maggots were in such abundance in the mouth and cloaca that they could not be removed from the sample; they were added to the vials

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with the feces and saliva. For carcasses with no moist fecal or saliva sample, small metal scissors were used to clip any available dried internal tissues around the mouth and cloaca. These tissues were placed in the vials. Most vials contained a mixture of fecal, saliva, and tissue samples. Samples were shaken by hand for approximately 60 seconds for homogenization. From this point, we followed manufacturer's directions, having replaced mosquitoes with fecal, saliva, and tissue samples. Indicator strips were inserted into the solution and interpreted in the field after 15 minutes.

Samples were stored at –80°C after we performed the test. To confirm the results, we used RT-PCR TaqMan to detect the presence or absence of WNV-RNA in these samples by a method similar to that used by Lanciotti et al. (6). A WNV strain (NY99) was used as a positive control. Alternatively, VecTest results were confirmed by IHC testing of the brain, heart, kidney, and spleen of the crow carcass from which the samples were derived. The IHC testing was conducted by the University of Illinois Veterinary Diagnostic Laboratory, according to the method outlined by Heinz-Taheny et al (7).

Results

We used VecTest to test all 20 crow samples; all indicator strips developed control lines, which indicated that the test had performed according to instructions. Nineteen samples were positive for WNV with a faint-to-bold WNV line; one sample was negative for WNV. IHC testing was performed on the crow carcasses from which five of these positive samples were derived, and IHC labeling for WNV antigen was present in all five of these samples, indicating 100% confirmation of the VecTest results. The remaining 15 vials containing the VecTest crow sample, composed of 14 positives and 1 negative, were assayed by RT-PCR TaqMan. Results from TaqMan showed 11 positives and 4 negatives (Table). In total, 17 (85%) of 20 of the VecTest results were confirmed with either IHC or RT-PCR TaqMan, and 3 (15%) of 20 involved conflicting results between the two testing methods for the samples. No significant difference existed between the positive and negative rates of VecTest and RT-PCR in a chi-square analysis of a 2x2 contingency table (chi square = 2.16, df=1, p=0.14). Using RT-PCR as the standard criterion, we found that VecTest results included three false positives, yielding a false-positive rate of 75%.

Table. Results of West Nile virus testing of American Crow fecal, saliva, and tissue samples

Sample no.	VecTest[a]	RT-PCR TaqMan[b]
1	+	+
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
7	+	+
8	+	+
9	+	-
10	+	-
11	-	-
12	+	+
13	+	+
14	+	+
15	+	-

[a]VecTest West Nile virus/Saint Louis encephalitis virus Antigen Panel Assay (Medical Analysis Systems Inc., Camarillo, CA).
[b]Reverse transcriptase polymerase chain reaction; Taqman (Applied Biosystems, Foster City, CA).

Conclusions

On the basis of their trials with mosquitoes, Ryan et al. (5) suggested that VecTest should not produce false-positive results. Although the rates of positives and negatives did not differ between VecTest and RT-PCR in this study, this result may be an artifact of small sample size. The false-positive VecTest results on American Crow fecal, saliva, and tissue samples suggest a low specificity; therefore, we recommend that VecTest be considered experimental in its application to dead American Crows until more extensive investigations are conducted. All positive VecTest results should be verified with another test. VecTest may be useful in early season screening, when rates of positives are typically low, or in nonpeak areas. We conducted this study in mid- to late summer in east-central Illinois, during a time when death rates of free-ranging American Crows were high.

RT-PCR detects genomic sequences of WNV, whereas VecTest detects the viral capsid. Because of the abundance of environmental RNAase and the possibility of the WNV capsid's persisting longer than the RNA, the three false positives detected by VecTest may indeed contain WNV capsid. Alternately, the conjugate in VecTest may react with a nonspecific protein in the fecal, saliva, or tissue samples of the dead crows to create a false positive.

This preliminary investigation establishes the basis for more comprehensive research on the full capabilities of this test for WNV detection in birds, including the sensitivity of the test, the postmortem period for which the test is viable, and the effectiveness across a range of species. We suggest that VecTest may be a cost-effective, rapid field technique for WNV detection in dead American Crows in the early transmission season or in areas with low transmission rates, although confirmation of positives is suggested at this time. This assay may be a useful tool for epidemiologic studies of WNV transmission cycles involving American Crows and will help to provide an epidemiologic basis for vector control efforts, although further study is warranted.

Acknowledgments

We thank Arlo Raim, Adam Arnold, Gabe Hamer, and Chris Warner for aid in capturing and tracking crows; Nina Krasavin and Hyun-Young Koo for laboratory assistance; John Andrews for interpreting immunohistochemistry results; and Jane Chladny and the necropsy and histology technicians at the University of Illinois Veterinary Medicine Diagnostic Laboratory for immunohistochemistry testing.

This research was supported by McIntire-Stennis Forestry Research Act Project, Centers for Disease Control and Prevention Grant U50/CCU520518-02 (RJN) and Department of Natural Resources Illinois Waste Tire Fund (RJN).

The authors declare no financial interests in the producers of VecTest.

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Severe Histoplasmosis in Travelers to Nicaragua

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We investigated an outbreak of unexpectedly severe histoplasmosis among 14 healthy adventure travelers from the United States who visited a bat-infested cave in Nicaragua. Although histoplasmosis has rarely been reported to cause serious illness among travelers, this outbreak demonstrates that cases may be severe among travelers, even young, healthy persons.

Histoplasmosis is a systemic infection caused by the dimorphic fungus, *Histoplasma capsulatum*. Infection results from inhaling spores, usually through exposure to bat and bird droppings in barnyards and caves. Although outbreaks have occurred after visits to bat-infested caves, histoplasmosis has not been frequently recognized as travel-related and has rarely led to serious illness among young, healthy travelers. This fungus is endemic in the United States along the Ohio and Mississippi River valleys and many other parts of the world, particularly Latin America. Histoplasmosis is often asymptomatic in endemic settings, but infection can result in a spectrum of illness, ranging from mild influenza-like illness to acute pulmonary infection and disseminated extrapulmonary disease. Immunocompromised persons and the elderly are at greater risk for disseminated disease (1). This report describes a recent outbreak of histoplasmosis among U.S. adventure travelers to Nicaragua that was associated with a high attack rate and hospitalizations among previously healthy travelers.

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The Study

In June 2001, five persons with a febrile respiratory illness visited a community hospital. The patients were among 15 persons (age range 19–61 years; median 38 years), residents of four states, who had participated in a geology-biology community college class trip to Nicaragua. To determine the cause and describe characteristics of the outbreak, we interviewed trip participants and reviewed medical records to collect demographic information, clinical history, and activities during the trip. Based on the partcipant's exposure to a cave with bats and clinical characteristics suggestive of acute histoplasmosis, diagnostic testing for histoplasmosis was conducted.

A case of acute histoplasmosis was defined as illness in a person who tested positive by one of the following laboratory tests: serology, urine antigen, histopathology, or culture. Immunodiffusion and complement- fixation serologic tests were performed at the Centers for Disease Control and Prevention (CDC). The immunodiffusion test was considered positive if an H or M band or both were detected. A complement-fixation titer to the yeast or mycelial antigens of $\geq$1:32 or a fourfold increase in the titer between acute- and convalescent-phase serum specimens was considered evidence of acute infection. Acute-phase serum specimens were obtained 2–3 weeks after exposure. Convalescent-phase serum specimens were collected approximately 3 months after exposure. One patient underwent bronchoalveolar lavage and bronchoscopy, and a biopsy specimen was obtained. Lung tissue was stained with hematoxylin and eosin and Gomori methenamine silver stains. Bronchoalveolar lavage washings were cultured by conventional methods (2). Identification of the organism as *H. capsulatum* was confirmed by GenProbe assay (GenProbe, San Diego, CA). Urine antigen tests, which were performed 2 weeks after exposure, are considered positive if $\geq$1 unit is detected (3,4).

The travelers began their trip on May 19 and returned to the United States on May 30. During their trip, they explored rock quarries, visited a biological research station, swam in freshwater lakes, and were exposed to parrots and insects. On May 21, a total of 14 of the 15 travelers entered a small cave that had previously been used as a silver mine. They remained in the cave for 10 minutes, during which time they saw several flying bats and bat guano on the ground.

The infection rate was 100% among the 14 travelers who entered the cave, and 12 (86%) were symptomatic. All 14 patients had serologically confirmed infection. Of the 14 patients, 12 (86%) had urine antigen tests (Table). The only traveler who did not enter the cave tested negative by serology and urine antigen.

Of the infected travelers, 8 (57%) were female. The average incubation period was 11 days (range 10–12 days),

Table: Summary of laboratory testing results and clinical outcomes among persons with histoplasmosis

Characteristic	N (%)
Diagnostic test result[a]	
CF[b] and ID[c] positive	5 (36)
CF positive, ID negative	8 (57)
ID positive, CF negative	1 (7)
Urine antigen positive[d]	7 (58), range 1.2–4.6, median 2.8
Clinical outcomes[e]	
Missed work, school, or both[f]	10 (83)
Treated with itraconazole	9 (75)
Treated with steroids	7 (58)
Hospitalized	6 (50)
Duration of hospitalization	Median 6 d (range 2–11 d)
Duration of fever	Median 12 d (range 4–26 d)
Duration of symptoms	Median 42 d (range 16–210 d)

[a]Among 14 persons with histoplasmosis.
[b]CF: complement fixation.
[c]ID: immunodiffusion.
[d]Urine antigen tests were performed for 12 persons.
[e]Among the 12 symptomatic persons.
[f]Five persons missed 2 weeks–3 months of work, and five persons missed one semester of school.

assuming that exposure occurred on May 21. Among the 12 symptomatic persons, 12 (100%) had a fever of 38.9°C to 40°C, nonproductive cough, myalgias, fever, chills, night sweats, loss of appetite, and mild headache. Many had nausea and vomiting (83%), chest pain (58%), and arthralgias (58%). Six persons had mild to moderate respiratory distress, with oxygen saturation of 88% to 95%. On physical examination, all the travelers' lung fields were clear to auscultation, a feature consistent with histoplasmosis infection. The two asymptomatic persons had previous exposure to bats or caving. Among the symptomatic persons, we found no differences in severity of symptoms according to sex, prior exposure to spelunking, residence in a histoplamosis-endemic area, or activities while in the mine.

In accordance with clinical treatment guidelines, physicians decided to treat patients with itraconazole for 6 to 12 weeks; some patients also received steroids (5). No patients required discontinuation of therapy because of adverse effects.

Laboratory studies showed normal complete blood counts, serum chemistries, and renal function tests. Four persons had mild to moderate elevation in liver function tests (alanine aminotransferase, range 55–204; aspartate aminotransferase, range 48–153; and alkaline phosphatase, range 95–311). All 12 symptomatic persons had abnormal chest radiograph results with bilateral nodular infiltrates (Figure). Two persons had hilar adenopathy, and one had a small pleural effusion.

Hematoxylin and eosin–stained sections of the lung tissue from the biopsy showed macrophages and neutrophils in the interstitium; no granulomatous inflammation was observed. Gomori methenamine silver stain demonstrated budding yeasts compatible with *H. capsulatum*. Culture of the bronchoalveolar lavage fluid from this patient grew *H. capsulatum* after 4 weeks of incubation.

Conclusions

This report highlights the importance of histoplasmosis as a potentially serious travel-related illness. Histoplasmosis is usually considered a mild, self-limited illness; however, this outbreak of histoplasmosis among previously healthy travelers was associated with a 100% infection rate of histoplasmosis and an 86% rate of symptomatic infection in the persons who entered the cave. Among the symptomatic persons, 50% required hospitalization and 83% missed school or work (range: 2 weeks–3 months) as a result of their illness (Table). Because of the high attack rate and the large percentage of patients with severe infection, these travelers were likely exposed to very high concentrations of *H. capsulatum* spores during their brief visit inside the cave.

H. capsulatum exists throughout the world (1,6); however, nonimmune travelers from areas with a low prevalence of histoplasmosis, who engage in high-risk activities in disease-endemic areas, are at greater risk of acquiring symptomatic fungal infection. The number of outbreaks of histoplasmosis, especially among U.S. travelers to Latin America, has increased. Histoplasmosis has been reported in groups of travelers who entered caves with bats in Costa Rica (7; unpub. data, Centers for Disease Control and Prevention (CDC), Ecuador (8), and Peru (9). In 2001,

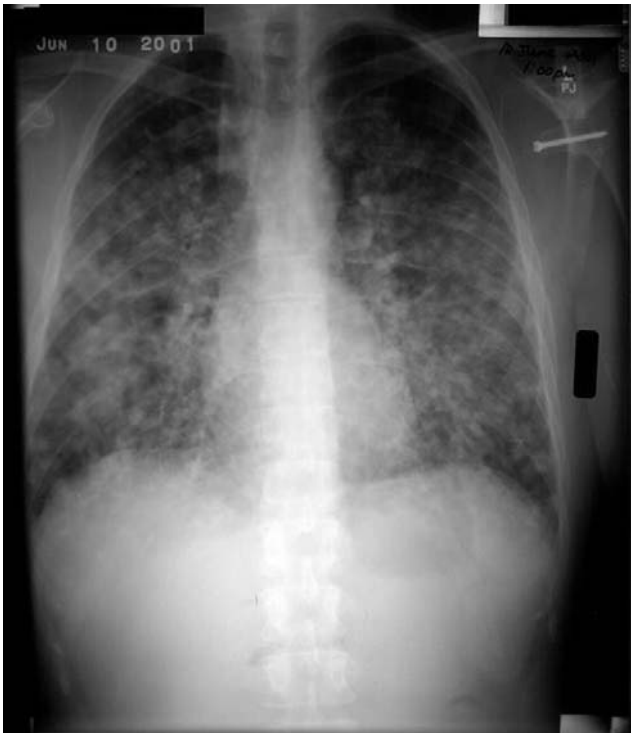

Figure. Chest radiograph of patient who acquired acute pulmonary histoplasmosis after visiting a cave in Nicaragua.

more than 200 college students became infected with histoplasmosis during a spring break trip to Acapulco, Mexico (10,11). Outbreaks and isolated cases of histoplasmosis have also been described among non–-U.S. travelers to Latin America and other areas of the world (12–19).

Clinicians should consider fungal pathogens when evaluating returning travelers who have a febrile respiratory syndrome. The differential diagnosis of acute febrile respiratory illness in international adventure travelers is extensive and includes legionellosis, psittacosis, leptospirosis, schistosomiasis, histoplasmosis, coccidioidomycosis, influenza, parainfluenza, mycoplasma, dengue, and malaria.

The timely diagnosis of histoplasmosis in travelers may be particularly challenging for clinicians evaluating one sporadic case or a patient involved in an undetected multistate outbreak. Antibody response can take 4–6 weeks to develop, reducing its usefulness in the acute setting and requiring the collection of a convalescent-phase specimen for confirmation. Antibodies may also persist for 5 years, making it difficult to distinguish between prior and recent infection without obtaining a convalescent-phase specimen. The sensitivity of immunodiffusion is 72% to 85%, while that of complement fixation is 80% to 90% (20,21). The antigen test can be used for urine, cerebrospinal fluid, serum, and bronchoalveolar lavage fluid, and results are available in 24 hours. This test had been used most extensively in AIDS patients; however, it is less sensitive in nonimmunocompromised persons with acute pulmonary infection. Sensitivity of the urine antigen test for detection of acute pulmonary infection ranges from 44% to 75% and increases when performed early after the onset of symptoms (3,4).

In this outbreak, 80% of the travelers were prescribed appropriate malaria chemoprophylaxis during a pretravel assessment. However, most clinicians may not have thought to counsel them about histoplasmosis risk and prevention. During pretravel visits, clinicians should review patients' itineraries for possible fungal exposures and should counsel them to avoid entering caves, especially those known to be bat infested. Since travelers may not know whether their itinerary includes visits to bat-infested caves, adventure travelers and persons who are at high risk for severe histoplasmosis infection, such as immunocompromised travelers (especially persons with AIDS), should be counseled to avoid possible exposures, either by avoiding caves or by using special protective masks. The National Institute for Occupational Safety and Health has published guidelines for special masks that can be used to reduce occupational and other exposures (e.g., among spelunkers). Unfortunately, these special masks are bulky and may be impractical for many travelers. (22). While most nonimmunocompromised patients with acute pulmonary histoplasmosis improve without treatment, persons with hypoxemia, diffuse pulmonary histoplasmosis, or severe illness requiring hospitalization may benefit from antifungal therapy and, in some cases, corticosteroids (5).

The contribution of histoplasmosis to infectious disease illness in travelers is likely underestimated. As adventure travel becomes increasingly accessible, especially among persons at high risk, immunocompromised persons, and the elderly, histoplasmosis and other fungal infections in travelers may become more common. Enhanced surveillance for histoplasmosis and other fungal infections by public health officials, combined with heightened awareness by clinicians who are evaluating symptomatic, post-travel patients, can lead to greater understanding of the epidemiology of fungal infections among travelers and, ultimately, to improved prevention measures.

Acknowledgments

We thank Juan Jose Amador of Nicaraguan Ministry of Health, Phyllis Kozarsky, the members of the group who participated in this trip, and the Histoplasmosis Reference Laboratory, which performed the urine antigen testing, for their help with this investigation, and Ava Navin for editorial assistance.

When the work was done for this outbreak investigation, Ms. Connolly was associated with Indiana University. She is currently an employee of MiraVista Diagnostics, Indianapolis, Indiana.

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Mayaro Virus in Wild Mammals, French Guiana

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A serologic survey for Mayaro virus (*Alphavirus, Togaviridae*) in 28 wild nonflying forest mammal species in French Guiana showed a prevalence ranging from 0% to 52% and increasing with age. Species active during the day and those who spent time in trees were significantly more infected, results consistent with transmission implicating diurnal mosquitoes and continuous infectious pressure.

The Mayaro virus is a zoonotic Alphavirus (family *Togaviridae*) responsible for epidemics of febrile exanthematous illness in Latin America and Amazonia (1). Although the death rate is low, Mayaro fever is a major arboviral infection relevant to public health in rural populations, with an increasing incidence of human cases in the Amazonian basin following ecosystem disturbances (2). The activity of the Mayaro virus can be described as a constantly moving wave, transmitted among susceptible vertebrates by sylvatic *Culicidae* mosquitoes (3). *Haemagogus* mosquitoes are the main vectors; they are diurnal canopy dwellers common in high pristine rainforests (4). Several vertebrate hosts, mainly primates, rodents, and birds, are considered to be reservoirs, although their exact role in maintaining the virus is insufficiently understood (2). Previous serologic surveys have shown high prevalence rates in primates, but the virus has been isolated only from lizards and one marmoset (5,6); experimental inoculation of marmosets resulted in a short period of viremia, although the titer was nevertheless probably high enough to infect vectors (4). Human disease outbreaks could occur when birds or vectors introduce the virus into rural areas with high densities of both *Haemagogus* and potential reservoirs.

This emerging disease has recently been reported in French Guiana, a French administrative unit on the northern coast of South America (6). To investigate the diversity of the reservoir species, a serologic survey for Mayaro virus was conducted on 28 nonflying mammalian rainforest species, in a total of 579 animals; no previous surveys in wild vertebrate species has included so many samples. Investigations on antibody responses in potential hosts are important first steps for understanding viral dynamics (7). Since the infectious process plays a major role in wildlife ecology (8), we used Mayaro infection as a case study to investigate the correlation between ecologic and biologic patterns of potential hosts and their susceptibility to infection.

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The Study

Blood samples were collected in 1994–95 during a wildlife rescue operation at the Petit Saut hydroelectric dam site (4°55′ N, 53°05′ W), French Guiana (9). The overall habitat of all species was pristine high rainforest. Each serum sample was tested by hemagglutination inhibition (HI) for antibodies to Mayaro and Tonate (Venezualan equine encephalitis complex) viruses. Serum samples with titers $\geq$1:20 were confirmed by seroneutralization at a 1:20 dilution (10). Briefly, equal volumes of diluted serum were mixed with a Mayaro virus suspension containing 100 tissue culture infectious dose 50 per 0.1 mL. The reaction was incubated at 37°C for 1 h. As control of the test dose of virus, the working dilution was successively diluted 10-fold. One hundred microliters of the serum-virus mixture, control virus, and diluted serum was inoculated in duplicate in monolayers of Vero-E6 cell line and incubated at 37°C for 5 to 7 days. A positive reaction by neutralization was considered with the total inhibition of the cytopathic effect in the cell monolayer induced by Mayaro virus. Analysis of variance ($p<0.05$ considered significant) was used to study correlations between ecologic patterns and arcsine-transformed seropositivity of species (XlStat-Pro, Addinsoft, Paris, France). The following ecologic parameters were considered: vertical use of space (arboreal, terrestrial, and both); density (low <10 individual animals/km^2; medium 10–20 individual animals, and high >20 individual animals/km^2); rhythm of activity (strictly diurnal, strictly nocturnal, or both); and lifespan. Because lifespan data in the wild are not available for most species, we used last reproduction age as an indicator, and we classified lifespan as short (last reproduction age <5 y), intermediate (last reproduction age between 5 and 15 y) , and long (last reproduction age >15 y). The data used are summarized in Table 1.

All the serum samples were negative for Tonate virus, which suggests that no cross-reaction occurred with Mayaro virus. The possibility of cross-reaction with Una virus, a closely genetically related Togavirus, was not considered since Una is known only in subtropical areas (11) and open habitats (12,13). The seroprevalence rates of Mayaro virus, on the basis of seroneutralization confirmation, ranged from 0% to 52% and reached 80% in two species (Table 2); however, only a limited sample was available. Gender had no apparent effect on the frequency

Table 1. Ecologic data used in the multivariate analysis

Species	Vertical use of space	Rhythm of activity	Lifespan	Density
Choloepus didactylus	A	ND	L	M
Tamandua tetradactyla	AT	ND	L	M
Bradypus tridactylus	A	ND	L	H
Dasypus spp.	T	N	I	H
Myoprocta acouchy	T	D	I	M
Dasyprocta leporina	T	D	I	H
Agouti paca	T	N	I	H
Coendou melanurus	A	N	I	M
C. prehensilis	A	N	I	H
Echimys spp.	A	N	I	M
Proechimys sp.	T	N	I	H
Mazama spp.	T	ND	I	L
Tayassu tajacu	T	D	I	M
Potos flavus	A	N	I	M
Nasua nasua & *Eira barbara*	AT	D	I	L
Didelphis marsupialis	AT	N	S	M
Didelphis albiventris	AT	N	S	M
Caluromys philander	AT	N	S	H
Metachirus nudicaudatus	T	N	S	M
Caluromys philander	AT	N	S	H
Alouatta seniculus	A	D	L	M
Saimiri sciureus	A	D	I	M
Pithecia pithecia	A	D	I	L
Saguinus midas	A	D	I	M

[a]Vertical use of space: A, arboreal, T, terrestrial, AT, both arboreal and terrestrial. Rhythm of activity: D, diurnal; N, nocturnal; ND, nocturnal and diurnal. Lifespan: S, short; I, intermediate; L, long (see text). Density: L, low; M, medium; H , high (see text).

of infection. The prevalence of infection increases with age in howler monkeys (14). A similar pattern has been observed in sloths, with only adults found to be seropositive. On the basis of hematologic and biochemical data, the virus infection had no apparent effect on the animal's health (14,15). Patterns of activity and vertical use of space were the two parameters with the greatest significant predictive value for positive serologic test results (p=0.03 and 0.01, respectively). The best analysis of variance model fitted these two variables (R^2=0.45, p<0.02). Species that

Table 2. Mayaro virus seroprevalence in free-ranging nonflying mammals, French Guiana (seroneutralization assay)

Order	Species (n)	Seroprevalence %
Xenarthra	2-toed sloth, *Choloepus didactylus* (26)	27
	3-toed sloth, *Bradypus tridactylus* (29)	3
	Kappler armadillo, *Dasypus kappleri* (20)	0
	Nine-banded armadillo, *D. novemcinctus* (40)	10
	Collared anteater, *Tamandua tetradactyla* (26)	23
Marsupiala	Common opossum, *Didelphis marsupialis* (29)	3
	White-eared opossum, *D. albiventris* (19)	10
	Brown 4-eyed opossum, *Metachirus nudicaudatus* (19)	0
	Grey 4-eyed opossum, *Philander opossum* (27)	19
	Woolly opossum, *Caluromys philander* (5)	20
Rodentia	Acouchy, *Myoprocta acouchy* (29)	0
	Red-rumped agouti, *Dasyprocta leporina* (29)	17
	Brazilian porcupine, *Coendou prehensilis* (26)	11
	Black-tailed porcupine, *Coendou melanurus* (15)	13
	Paca, *Agouti paca* (17)	0
	Terrestrial spiny rat, *Proechimys* sp. (18)	5
	Arboreal spiny rat, *Echimys* spp. (21)	5
Carnivora	Kinkajou, *Potos flavus* (9)	11
	Coati, *Nasua nasua* and Tayra, *Eira barbara* (7)	11
Artiodactyla	Collared peccary, *Tayassu tajacu* (7)	0
	Brocket deers, *Mazama* spp. (10)	0
Primata	Red howler monkey, *Alouatta seniculus* (98)	52
	White-faced saki, *Pithecia pithecia* (5)	80
	Squirrel monkey, *Saimiri sciureus* (6)	67
	Golden-handed tamarin, *Saguinus midas* (42)	19

are active during daytime and arboreal or arboreal/terrestrial species were found to be more frequently infected than others primates of the *Cebidae* family (howlers, sakis, and squirrel monkeys); two-toed sloths had the highest prevalence. Prevalence rates were highly variable among species exhibiting only one of the explicative patterns; and no species that is strictly terrestrial and nocturnal, such as the four-eyed opossum, the spiny rat, and the paca, was infected (Figure).

Discussion

Ecological dynamics are often not included in the epidemiology of Amazonian diseases; the diversity of arthropods and vertebrates and their ecological conditions and the difficulty in obtaining samples have resulted in limited understanding of arboviral infection patterns. This survey of the Mayaro virus in the French Guiana rainforest was based on a large number of individual animals and a wide variety of nonflying mammalian species with different ecologic habits. The survey corroborated previously reported epidemiologic patterns while providing some additional features. No bird, bat, or reptile species was included in the survey, since the wildlife rescue operation to collect samples was not focused on such species. But although limited to nonflying mammals, the multivariate analysis shows that arboreal or diurnal species are infected significantly more frequently than others. Thus, most hosts are bitten while in the upper forest layer and when foraging at the same hours as the vectors. The previously reported infection of sloths and howler monkeys (5) is explained by our findings, as their way of life comprises the two factors found to be linked to infection. Mayaro infection has also been reported in agoutis (5): infection of these strictly terrestrial diurnal species may reflect the ecological plasticity of the vector or may indicate that other mosquitoes, with different ecologic patterns, are implicated in transmission. The fact that lifespan and density do not contribute to the variation in seroprevalence may reflect the ecological ubiquity of the vectors and the fact that the virus circulates easily in both the vectors and the host populations (16). Increasing seroprevalence with age has been also described in sloths with St. Louis encephalitis virus (17) and could also be related to constant activity of the arbovirus and infectious pressure. Comparative seroprevalence surveys in areas with different host diversity and density would be of interest.

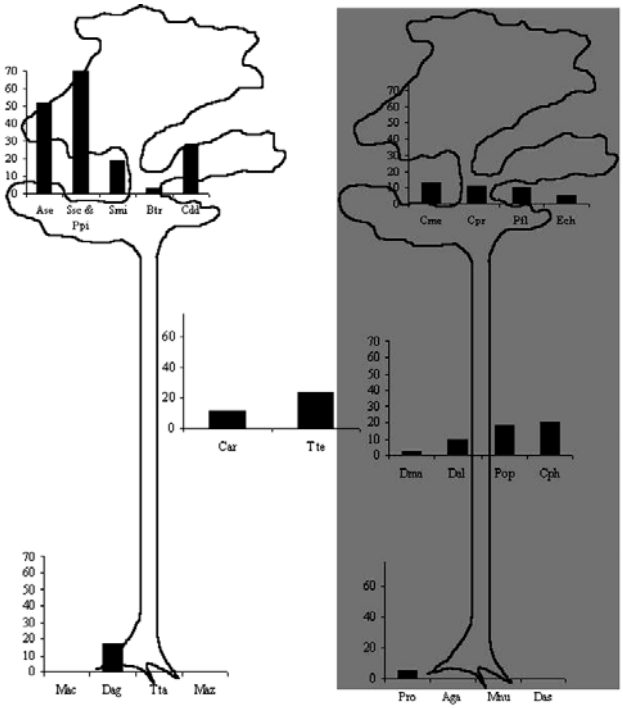

Figure. Mayaro virus seroprevalence rates in Neotropical mammal species, according to 1) activity period of animals (diurnal species on the left, nocturnal species on the right, diurnal and nocturnal species in the center); 2) vertical use of space (terrestrial, arboreal, or both). Ase, *Alouatta seniculus*; Smi , *Saguinus midas*; Ssc, *Saimiri sciureus*; Ppi, *Pithecia pithecia*; Btr, *Bradypus tridactylus*; Cdd, *Choloepus didactylus*; Tte, *Tamandua tetradactyla*; Car, carnivores (*Nasua nasua* and *Eira barbara*); Cme, *Coendou melanurus*, Cpr, *Coendou prehensilis*; Ech, *Echymis* spp.; Pfl, *Potos flavus*; Dma, *Didelphis marsupialis*, Dal, *Didelphis albiventris*; Pop, *Philander opossum*; Cph, *Caluromys philander*; Mac, *Myoprocta acouchy*; Dag, *Dasyprocta leporina*; Tta, *Tayassu tajacu*; Pro, *Proechimys* sp.; Apa, *Agouti paca*; Mnu, *Metachirus nudicaudatus*; Das, *Dasypus* spp., Maz, *Mazama* spp.

Some strong differences were observed between species with ecologic patterns favorable to infection. For example, the two-toed sloths and large primates were more frequently infected than three-toed sloths, carnivores, and tamarins. These differences may be linked to microhabitat use, behavioral patterns, or specific sensitivity. Although the geometric HI mean titer and seroprevalence were not significantly linked (i.e., species with greater exposure appeared to have lower titers), this finding could be related to continual exposure to virus infection risk and a latent or chronic infection (18). On the contrary, animals from species less exposed to infection (kinkajous, four-eyed opossums, arboreal porcupines) showed higher titers, probably because of accidental infections resulting in intense immunologic response. However, more data on virus isolation and experimental infections are necessary to confirm those assumptions.

Information on the incidence of disease and the pathogenicity of infectious agents in wildlife is still limited. Although the susceptibility of a host species remains speculative when the agent has not been isolated, a positive antibody response shows that a specific antigen, or a serologically closely related antigen, is or was present, and that the infected species has been exposed and has responded. Serologic investigations in free-ranging species are often

limited to transversal surveys in several animals at a single time. The levels and distribution of seropositivity and titers can, however, be used for a better understanding of both virus dynamics and host susceptibility. In infections with alphaviruses, the viremia is intense but short (7), but the diversity of the potential reservoirs may compensate for this short period. When the virus is introduced into a new area, many individual animals can be infected rapidly. The low individual excretion rate is compensated for by the number of animals possibly affected by the virus. This viral strategy is completely opposite to that of the *Arenaviridae*, where the reservoirs are much less diverse (usually a single species), but the hosts occur at high density and viral excretion is long-lasting (19). Other arboviruses with an ecologic niche similar to that of the Mayaro virus may also have a wide diversity of potential reservoirs. The Mayaro virus is active mainly in forests, but *Haemagogus* spp. can fly over large areas. Howler monkeys, tamarins, squirrel monkeys, and agoutis are also still common in the vicinity of the main cities of French Guiana, and periurban species may also be infected and act as reservoirs. Moreover, alphaviruses are prone to be hosted by a large range of vectors (3) and the risk for new epidemic patterns, comparable to the emergence of periurban Chikungunya cycle in Africa, should be considered (1). Demographic pressure is resulting in increasing human contact with forest habitats and fauna; the predictable health consequences of this evolution have been emphasized in parasites (20) and may also be applicable in arboviruses.

Acknowledgments

We are grateful to J. Lelarge and E. Bourreau for technical help.

The serum samples were collected during the Programme Faune Sauvage, funded by Electricité de France–Centre National d'Equipement Hydroélectrique.

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The European Commission's Task Force on Bioterrorism

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In response to the increased threat of bioterrorism, a task force on health security was established in the European Commission. Task force members address a broad range of issues related to preparedness for and response to bioterrorist events and seek to bring about a greater collaboration between the European Union member states.

The deliberate release of anthrax in the United States of America in September and October 2001 completely changed the international perception of the risk of bioterrorism. Before that time, public health preparedness for bioterrorism was not a political priority in the European Union (EU) member states. Preparedness and response plans and actions have since been given a higher priority on EU member agendas. Bioterrorism also initiated a discussion on the need to improve preparedness through reinforcing existing public health structures that are in charge of monitoring and controlling diseases.

On October 19, 2001, at the European Council in Ghent, Belgium, the Council of Ministers of the EU and the European Commission (EC) were asked by the heads of state and government to prepare a program to improve the cooperation between member states for the evaluation of risks, alerts, and intervention, the storage of the necessary means, and the collaboration in the field of research.

In parallel with these developments at the European level, many well-focused national activities were also carried out. Countries reexamined their preparedness plans and adapted them to the new threats, and legislation needed to implement countermeasures was reviewed. The national authorities also discussed the necessity of stockpiling medicines and other medical supplies and most upgraded their stocks of vaccines and antibiotics, especially smallpox vaccine. To improve the ability to recognize a deliberate release, countries developed training programs for different kinds of first responders.

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On the initiative of the European Commissioner for Health and Consumer Protection, the Health Security Committee (HSC) was set up with representatives of the ministries of health from each member state. Following a proposal by the commission, the HSC agreed on December 17, 2001, on a program for cooperation on preparedness and response to biologic and chemical agent attacks (or Health Security Programme). The program comprises 25 actions grouped under four objectives (available from: URL: http://europa.eu.int/comm/health/ph_threats/Bioterrorisme/bioterrorisme_en.htm). The overall goal is to improve cooperation between the member states with the aid of the commission and to facilitate collaboration between the different national authorities involved in public health preparedness for bioterrorism. During this process, gaps in resources and methods are identified and projects initiated to fill these gaps. As is standard practice in many sectors of activity at EU level, existing examples of preparedness in the member states are shared and can be extended or adapted for use throughout the EU. Projects and activities under the health security program are funded through existing funds, especially from the budget foreseen for the EU's public health programs. Other funds such as from the sixth framework program for research could also be used for activities of relevance to the health security program.

Since the beginning of May 2002, a 15-member strong task force has been set up by the Directorate-General of Health and Consumer Protection, comprising nine national experts seconded from different institutions in the EU countries and six commission officials. The task force includes representatives from the Robert Koch Institute and the Paul Erhlich Institute (Germany), the Scientific Institute of Public Health (Belgium), the Swedish Institute for Infectious Diseases Control (SMI, Sweden), the National Institute of Public Health and the Environment (RIVM, Netherlands), the Hellenic Institute for Infectious Disease Control (Greece), the Italian National Institute for Health (Italy), as well as the Pitié-Salpêtrière Hospital in Paris (France). The task force's main objective is to implement the health security program. To this end, members have initiated further activities with key players in the member states, some of which are described below.

One of the initial priority activities was the setting up of a mechanism for rapid information exchange, consultation, and coordination on all aspects dealing with bioterrorism. All designated authorities of the EU member states have now been connected through this network by means of email, fax, and telephone. This tool makes it possible for the task force and the nine member states to disseminate information rapidly about suspected or confirmed incidents and for national authorities to evaluate measures planned with their implications for EU and for other mem-

ber states. The task force is available 24 hours a day, 7 days a week to facilitate the process. By May 2003, the system had been used in two major EU-wide exercises and for nine alerts about suspicious events communicated by the member states.

The early detection of a deliberate release or bioterrorist attack is an essential part of a program of preparedness. One essential tool to make this possible will be the regular surveillance activities already in place at national and European level. Certain modifications to these systems, such as the addition of certain diseases considered as having a high potential for deliberate release, would have to be done to make the systems better adapted to the new challenges of bioterrorism. To achieve this, the Community Network on Communicable Diseases (the already existing mechanism for EU collaboration in the areas of infectious disease surveillance and control) extended its list of diseases to be reported, to include additional threats such as tularemia, anthrax, Q fever, and smallpox.

When the task force was established, several lists of agents that could be involved in a biologic attack and considered of major bioterrorist threat had been established and published. Against this background, an approach more suitable to the demands for focused action in Europe was developed though a matrix model designed to provide an evaluation of the public health impact of any given agent that could be used in bioterrorism. The matrix model may also be used to identify areas where the public health response needed strengthening for any given agent. This means that for a given type of action a different set of agents would emerge as deserving the highest attention. The matrix model is currently being validated and has produced lists targeting specific areas of public health preparedness, which are currently being reviewed by members of the HSC. Using this model, identifying the public health impact of new threats should be possible, such as severe acute respiratory syndrome (SARS) (once it has been better characterized), multidrug-resistant tuberculosis, or any other new or drastically changed disease-causing agent.

The clinical management, including recognition, of many of the diseases caused by the bioterrorist agents is likely to be difficult, since they are, to a large extent, unknown to the average clinician in Europe today. To provide clinicians with guidance and a source of generally agreed-upon information, 10 different articles on 48 major agents have been compiled (*Bacillus anthracis* (anthrax), *Yersinia pestis* (plague), *Francisella tularensis* (tularemia), smallpox and monkeypox viruses, *Clostridium botulinum* (botulism), hemorrhagic fever viruses, encephalitis viruses, *Coxiella burnetii* (Q fever), *Brucella* species (brucellosis), *Pseudomonas mallei* (glanders), and *Pseudomonas pseudomallei*). They have been sent for comments to experts in all member states, have been adopted by HSC, and will be made available to the European medical community. Their publication in a peer-reviewed medical journal is currently being considered.

The laboratory capacity required to diagnose most of the potentially toxic and infectious agents that might be used in case of deliberate release is believed to exist within the EU as a whole; however, no member state has the complete spectrum of the diagnostic capabilities needed. To improve the collective preparedness and to make more efficient use of the available resources, the laboratory capacities already in place are currently under review. As a long-term goal these capabilities would be made available to all member states of the EU. Additionally, more concrete collaboration between laboratories was initiated to share techniques and participate in common quality assurance schemes. Assisted by the task force, the P4 (U.S. BSL-4) laboratories in the EU are currently forming a network that will enhance their research activities through common projects. They have been asked to develop procedures to make P4 diagnostics available to all member states and to handle, under high safety level requirements, a substantial workload of analysis of environmental samples that might arise following a series of bioterrorist incidents or threats.

The European Agency for the Evaluation of Medicinal Products (EMEA) has already developed guidelines on the use of authorized pharmaceuticals in case of deliberate release of biologic and chemical agents (available from: URL: http://www.emea.eu.int/index/indexh1.htm). This includes guidelines for treatment and prophylaxis of the agents on the so-called CDC A-list of the most important threats as bioterrorist agents (1).

Stockpiling of medicines and vaccines (in particular, smallpox vaccines) has been extensively discussed between the different member states and the EC. Although the member states initially looked into the possibility of an EU-level vaccine stockpile, the member states opted to establish national stockpiles sufficient for their projected needs.

Smallpox has become the model for much of the work performed in the area of preparedness for bioterrorist incidents. Vaccination is certainly an essential countermeasure against possible smallpox threats, yet finding the right balance under different vaccine administration scenarios between personal and societal cost-benefit ratios has proved extremely difficult. To find optimal solutions, various attempts have been made to construct appropriate mathematical models. Currently a group of European modelers has been called by the task force to work together in a network to develop models for EU purposes. In addition, this group has been asked to give expert opinions on new models published and to establish an infrastructure for

data-exchange that could be used for real-time modeling, should the need arise.

Many member states have developed (or are in the process of developing) specific preparedness plans for smallpox. The task force has put in place a compilation and review process, which includes the tabulation and comparison of these plans to improve the understanding of how and with what measures individual countries respond to specific triggers and events and how components of their plans, which are important for the whole EU, will interact.

National authorities had already developed substantial capacities to deal with chemical incidents. Much of the EU work on chemical incidents has been done in close collaboration with other organizations like World Health Organization (WHO) and Organization for the Prohibition of Chemical Weapons. A special working-group has been formed under the auspices of HSC, and a project will be initiated to take work on certain key aspects forward.

In most areas investigated, existing European expertise was identified that could ensure a high level of preparedness against biologic or chemical agents. Considerable effort is needed, however, to determine the exact kind of expertise needed for specific cases and to identify specialists and develop the modalities required for effective sharing of this expertise between countries. As a first step, a plan for the development of a common directory of experts has been developed, and countries have been asked to make their national expertise available on behalf of the EU. Further modalities will be developed when this first step is implemented.

Better preparedness should also include improved coordination between the commission and international organizations working in this area such as the WHO and the parties to the Global Health Security Action Initiative (Canada, France, Germany, Italy, Japan, Mexico, the United States, the United Kingdom, and the European Commission with WHO participating as a resource). Multiple initiatives regarding coordination of actions in this area are being pursued.

The EU program on health security is expected to be concluded in November 2003. However, this deadline likely does not provide enough time to complete the activities anticipated in many of the areas included in the 25-point action program. An extension of the task force's duration is therefore being sought. In the areas where activities have taken place, a centrally launched initiative at EU level has been found to be beneficial both for the member states and for the EU as a whole. The work has opened new communication channels between member states, improving both the understanding of the preparedness in other countries as well as giving the possibility to learn from good practice and tried solutions. The EC has also gained experience in working with a group of experts who initiate activities with more operational effectiveness than has been the case in the past. This experience will be very useful in the continued discussion on the development of a European Centre for Disease Prevention and Control since the task force can be seen as a precursor to this centre, albeit in a narrow area of activity. During the remaining 6 months and the period of extension, if it is approved, the work in the area of bioterrorism will continue to strengthen the capabilities at EU level to respond to biologic and chemical threats. The capabilities to manage diseases in emergency situations in general will be improved, and knowledge gained will be most useful for other areas of emergency preparedness.

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Wild-type Measles Virus in Brain Tissue of Children with Subacute Sclerosing Panencephalitis, Argentina

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We studied eight children who had measles at 6 to 10 months of age during the 1998 Argentine measles outbreak and in whom subacute sclerosing panencephalitis developed 4 years later. We report the genetic characterization of brain tissue–associated measles virus samples from three patients. Phylogenetic relationships clustered these viruses with the wild-type D6 genotype isolated during the 1998 outbreak. The children received measles vaccine; however, vaccinal strains were not found.

Measles is often incorrectly regarded as a mild disease, and priority is not given to measles elimination programs in some countries (1). Nevertheless, substantial progress has been made in eliminating measles virus from the Americas through massive vaccination campaigns and maintaining high measles population immunity over time. From 1990 to 1996, measles cases declined from 250,000 to 2,109; however, in 1997, measles reemerged in the Americas, with 70,983 confirmed cases. From the last Argentine outbreak (July 1997–May 1999), 10,354 confirmed measles cases were reported, most of them in unvaccinated preschool-aged children in the greater Buenos Aires metropolitan area (2).

Although measles virus (*Morbillivirus* genus, *Paramyxovirus* family) is not highly neurotropic, it can establish long-term persistent infection in brain cells. Three different neurologic complications result from interactions of measles virus with neural tissue. Acute postinfectious encephalitis, usually appearing 5–14 days after the rash, is thought to be a virus-induced autoimmune disease. Measles inclusion–body encephalitis, which occurs in immunocompromised patients after a latent period of 3–6 months, is believed to be a direct measles virus infection of neural tissue. The third form, subacute sclerosing panencephalitis (SSPE), manifests 2–10 years after primary measles infection as a progressive and fatal chronic neurodegenerative disease caused by persistent defective measles virus in neurons and oligodendrocytes. SSPE develops with high titers of anti-measles antibodies both in cerebrospinal fluid (CSF) and serum, which seem unable to eliminate measles virus from the brain (3).

Although measles virus is serologically monotypic, genetic variability has defined eight clades, including 20 genotypes and a putative new genotype that are geographically and temporally restricted (4). Evidence does not indicate that wild-type measles virus strains differ in terms of either pathogenesis or neurovirulence. Measles virus recovered from patients with SSPE differs from wild-type viruses in a number of mutations that mainly affect the matrix, hemagglutinin (H), nucleocapsid (N), and fusion genes. The matrix accumulates the highest level of mutations in the entire open reading frame; by contrast, the N is modified in the carboxyl terminus, and the H has biased hypermutation in a limited region (5,6). Despite the paucity of studies in molecular epidemiology of SSPE, measles virus sequences obtained from brain tissues are homologous to the genotype circulating at the time of primary exposure to measles virus (7).

We studied eight children who had measles as infants during the 1998 measles outbreak in Argentina and in whom SSPE developed 4 years later. We report the genetic characterization of brain tissue–associated measles virus from three of these patients.

The Study

Diagnosis of SSPE was based on Dyken's criteria, which include progressive cognitive decline and stereotypical myoclonus, characteristic electroencephalogram (EEG) changes, raised CSF globulin levels without pleocytosis, raised CSF measles antibody titers, and typical histopathologic findings in brain biopsy materials (8).

Antimeasles immunoglobulin (Ig) G antibodies in CSF and serum samples were assessed by using an automated qualitative enzyme-linked fluorescent immunoassay (bioMérieux, Marcy l'Etoile, France) and a quantitative indirect immunofluorescence test (Bion, Des Plaines, IL). In three patients, measles virus RNA was isolated from a punch of white matter obtained in the course of Ommaya reservoir implantation at SSPE onset. RNA was purified by the guanidinium-thiocyanate-phenol-chloroform method, and genetic characterization of measles virus was performed by sequencing the 450 nt from the carboxyl terminus of nucleoprotein (N) gene and a 377-bp fragment of the H gene (9). Viral fragments were reverse-transcribed with omniscript and sensiscript enzymes, and amplified with Hot Start Taq DNA Polymerase (QIAGEN GmbH, Hilden, Germany). Purified polymerase chain reaction

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products were labeled with DyET terminators and analyzed in an automatic capillary DNA sequencer (Amersham Biosciences, Piscataway, NJ). Comparisons were made with reference measles virus strains (4). Amino acid sequences were inferred. Nonsynonymous versus synonymous mutations (ω) and transitions versus transversions (κ) ratios were calculated. Sequences were aligned with ClustalX software, while phylogenetic analysis was performed by distance methods with Phylip software package v. 3.5c (10,11). Sequences derived from this study were submitted to GenBank.

Discussion

Clinical, epidemiologic, and laboratory findings of eight SSPE patients are described in the Table. Mean age at SSPE onset was 54 months (range 40–75 months), and the mean lag period was 48 months (range 33–68 months). Most patients (six of eight) resided in the greater Buenos Aires metropolitan area, one from the northeastern region and the other from the northwestern region. All patients met at least clinical, EEG, and CSF antibody titer criteria defined by Dyken (8). In particular, patient 4 (at 6 months of age) had a clinical diagnosis of measles in September 1998 during the outbreak. Six months later, the patient received Measles Mumps Rubella (MMR) vaccine according to the official immunization schedules. For patient 5, clinical diagnosis of measles was made at 10 months of age in September 1998 during the outbreak. At that time, he also had varicella; 2 months later, the patient received the MMR vaccine. Patient 6 was born in November 1997 and had no previous history of measles infection. She also received antimeasles vaccine at 6 months of age during the measles outbreak when the vaccination age was lowered.

Molecular Findings

The 450 nt from the carboxyl terminus of N gene and a 377-bp segment from H gene from three SSPE cases were analyzed and amino acid sequences were inferred. When compared to Edmonston strain, not only were a higher number of mutations found in N gene (36 vs. 19) but also more replacement changes (18 vs. 12) and a higher κ ratio (2.1 vs. 1.2) as compared to H gene. However, we compared the ω ratio and found it to be higher for H gene (1.7 vs. 1). Fixed replacements were detected at position 357 in H protein and at position 467 in N protein. Variability was detected in both genes. Changes found in SSPE samples related to D6 genotype, and the consensus sequence from last Argentine outbreak are summarized in Figure 1.

Distance methods were applied, and the matrix rendered similar results for both analyzed genes. The tree was built with measles virus genotype reference strains and Argentine strains previously characterized from 1991 and 1998 outbreaks. Phylogenetic relationships clustered the three SSPE strains with the wild-type D6 genotype. SSPE samples were strongly associated with wild-type D6 samples from the 1998 outbreak, supported by a bootstrap value of 100 out of 100 pseudoreplicates done. Divergence from genotype D6 (NJ-1 strain) was <2.2% in the N gene and <2.4% in the H gene. The unrooted tree for the carboxyl terminus of N gene was plotted (Figure 2).

Conclusions

The measles vaccine was included in the regular immunization schedule in Argentina in 1978. Despite vaccination, several disease outbreaks have occurred (12). Although we had previously performed a thorough molecular description of acute measles outbreaks, genetic characterization of SSPE has not yet been reported in Argentina (9).

SSPE results in widespread destruction of brain tissue, including both gray and white matter. Infectious virus likely reaches the brain at the time of the original systemic spread of measles virus, where the virus becomes clonal, disseminating gradually throughout the nervous system from the point of entry (13). High levels of antimeasles antibodies are found both in serum and CSF, and their relative titers in the two compartments indicate intrathecal synthesis of immunoglobulins.

Measles virus in SSPE is characterized by a low expression of viral envelope proteins as a result of mutational

Table. Clinical, epidemiologic, and laboratory findings for eight pediatric subacute sclerosing panencephalitis (SSPE) patients[a]

Patient	SSPE1	SSPE2	SSPE3	**SSPE4**	**SSPE5**	**SSPE6**	SSPE7	SSPE8
Date of birth	Jan-98	Feb-98	Jul-98	Mar-98	Nov-97	Nov-97	Sep-96	Nov-97
Date of measles disease	Sep-98	Sep-98	May-99	Sep-98	Sep-98	N/A	Jul-97	Jul-98
Date of SSPE onset	May-01	Dec-01	Feb-02	Jun-02	Aug-02	Sep-02	Jan-03	Feb-03
Geographic location	GBA	GBA	NW	GBA	GBA	GBA	NE	GBA
CSF antibody titers	1:1600	1:1600	1:3200	1:3200	1:400	1:400	1:1600	1:400
Serum antibody titers	>1:3200	>1:3200	1:12800	>1:3200	>1:3200	1:12800	N/A	1:400
Initial diagnosis	Progressive myoclonic epilepsy	Acute disseminated encephalo-myelitis	Progressive myoclonic epilepsy	Neuronal ceroid lipofucsinosis	Lennox-Gastaut syndrome	Progressive myoclonic atonic epilepsy	Chorioretinitis	Ataxia

[a]Molecular studies were done for SSPE patients highlighted in bold type. N/A, not available; GBA, greater Buenos Aires metropolitan area; NW, northwestern region from Argentina; NE, northeastern region from Argentina; CSF, cerebrospinal fluid.

A-Nucleoprotein

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          346        356        366        376        386        396
A         SMGGLNFGRS YFDPAYFRLG QEMVRRSAGK VSSTLASELG ITAEDARLVS EIAMHTTEDK
D6        .......... .......... .......... .......... .......... .........R
SSPE1     .......... .......... .......... .......... .......... .........R
SSPE2     .......... .......... .......... .......... ....N.S... .........R
SSPE3     ..R....A.. .......... .......... .......... ......S... .........R

          406        416        426        436        446        456
A         ISRAVGPRQA QVSFLHGDQS ENELPRLGGK EDRRVKQSRG EARESYRETG PSRASDARAA
D6        .......... .......... .....G.... ....A..... ...D...... S.........
SSPE1     ...PF..... .......... .....G.... ....A..... .......... S.........
SSPE2     .......... .......... .....G.... ....A..... .......... S.........
SSPE3     .......... .......... .....G.... ....A..... .......... S.........

          466        476        486        496        506        516
A         HLPTGTPLDI DTASESSQDP QDSRRSADAL LRLQAMAGIS EEQGSDTDTP IVYNDR
D6        ....S..... .....T...L .......... .......... .......... R.....
SSPE1     .P..S..... .....T...L .......... .......... .......... ....--
SSPE2     .P..S..... .....T...L .......... .......R.. .......... R.....
SSPE3     .P..S..... .S...T...L .......... .......... .......... R.....
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B-Hemagglutinin

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          281        291        301        311        321        331
A         SNDLSNCMVA LGELKLAALC HGKDSITIPY QGSGKGVSFQ LVKLGVWKSP TDMQSWVPLS
D6        ...F...... .......... .......... .......... .......... ..........
SSPE1     .......... .......... .......... .......... .......... ..........
SSPE2     .......... .......... .......... .......... .......... ..........
SSPE3     -..F...... .......... .......... .E........ .......... ..........

          341        351        361        371        381        391        401
A         TDDPVIDRLY LSSHRGVIAD NQAKWAVPTT RTDDKLRMET CFQQACKGKI QALCENPEWA PLKDNR
D6        .......... ......I... .......... .......... .......... .......... ......
SSPE1     .......... ......I... .......... .......... .......... .......... .....G
SSPE2     .......... ......S... ........R. ....EQ..... ....H..... ......S... ......
SSPE3     .......... ......I... .....P.... .......... ....H..... .......... ......
```

Figure 1. Replacement changes found in N and H genes from subacute sclerosing panencephalitis patients. Comparisons were made with D6 genotype reference strain (New Jersey, USA/94/1). Sample Buenos Aires.ARG/21.98 was taken as a consensus sequence for the 1998 Argentine outbreak (ARG21.98). Numbers indicate the position in A-nucleoprotein and B-hemagglutinin protein, respectively. Dots designate the same residue as genotype D6. Nonsilent changes are represented by single letter amino acid code. Antigenic sites are underlined.

events. Among such proteins, H is an attachment protein that mediates binding to cells and contributes to cell-to-cell fusion. The V357I mutation was one of the fixed changes that we had detected in the 1998 outbreak, but one of the SSPE cases (SSPE5) had a nonconservative V357S change at the same position (from hydrophobic to polar without charge) (9). This finding indicates that this particular position may be under strong positive selection supported by a high ω=1.7 and a low κ=1.2. Although this fragment is a relatively small portion of the H gene, it contains a major antigenic surface determinant (aa368–396), which may be relevant for neurovirulence, as well as three linear epitopes containing conserved cysteines 381, 386, and 396. SSPE5 presented 5 replacement changes; one shared with SSPE6 (P368R, D374E, K375Q, Q384H, and P397S) (14,15).

On the other hand, the N protein is more closely linked to viral protein interactions and thus has less constraints to vary as demonstrated by a higher κ (2.1) and lower ω (1) denoting neutral selection. Nevertheless, the B-cell epitope (aa457–476) showed the conservative L467P change in SSPE samples as well as in 1998 outbreak samples, differing from the D6 reference strain (NJ-1) (16).

Molecular data from the 1998 Argentine outbreak showed that the virus belonged to the D6 genotype and that analyzed regions were highly homogeneous and almost identical to other D6 strains isolated in South America (9). Accordingly, a single chain of transmission could be responsible for the spread of the genotype from European countries to Brazil and then to Argentina (17). The last Argentine outbreak began in July 1997 in the northeastern region of the country, bordering on Brazil; had its peak in greater Buenos Aires metropolitan area in July 1998; and ended in the northwestern region bordering on Bolivia in May 1999. For that reason, a displacement in time of acute measles and SSPE onset can be observed in SSPE3 and SSPE7.

Children in whom SSPE developed were born during the last measles outbreak and reached 6 months of age when the outbreak was at its peak. They acquired measles when they were 8 months of age (range 6–10 months), and SSPE was not detected among patients >1 year of age at the time of acute infection; however, underdiagnosis is a

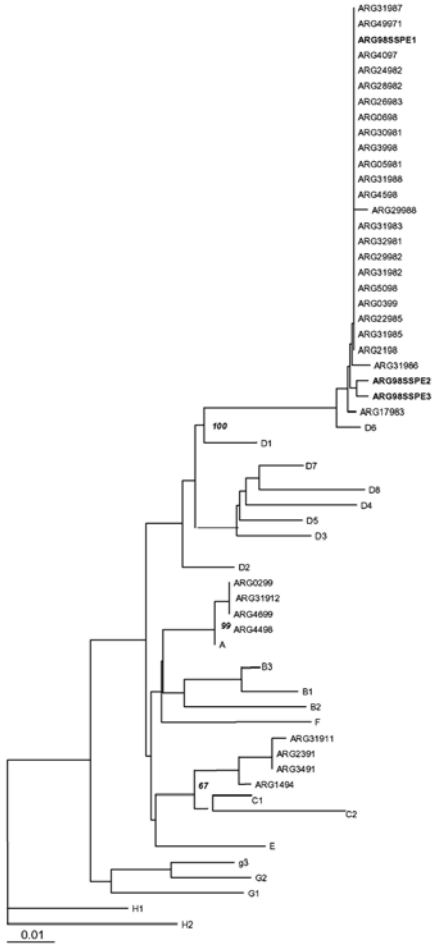

Figure 2. Phylogenetic relationships of subacute sclerosing panencephalitis (SSPE) strains. The neighbor-joining unrooted tree was plotted with Treeview 1.5.2. Reference measles virus strains are described (2). Wild-type (genotype C1 in 1991 and D6 in 1998) as well as post-vaccinal cases (genotype A) from the last two Argentine outbreaks were included (GenBank accession no. AF263841, 43, 44, 46, 52) (7). SSPE strains are highlighted in bold type (GenBank accession no. AY253332–37)

permanent challenge. Time lag between acute and SSPE onset was approximately 4 years, according to the reported data, but the rate for developing SSPE was higher than previously described (8).

Phylogenetic analysis of three SSPE cases from the last outbreak clustered with D6 genotype that circulated in Argentina in 1998. Although the original sequence of the wild-type virus that caused acute infection is unknown, we have a consensus sequence that summarizes the outbreak; therefore, we infer that changes may have occurred since then and contributed to the development of SSPE. Although all three patients had been immunized according to the schedule, vaccinal strains were not detected in brain tissue. These results agree with those recently reported for SSPE patients in the United Kingdom and Papua, New Guinea (18,19). Our data show that these three patients had been infected with wild-type circulating D6 virus before immunization. This primary measles virus infection in nonimmunized infants may be the leading cause of the high rate of SSPE inferred from our data. After brain tissues of deceased adults were randomly autopsied, Lawrence et al. raised the possibility that brain measles infection does not invariably lead to neurologic disease caused by measles virus. Therefore, neurologic disease mediated by measles virus may depend on the primary infection age (20). Although the basis for measles-associated neurologic disease is unclear and more thorough molecular studies need to be performed, our findings contribute to worldwide efforts in molecular characterization of SSPE strains and aim to increase awareness among physicians to improve diagnosis at early stages.

A.S.M is a member of Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC). P.R.B. and M.V. are fellows from the National Council of Investigation and Technology (CONICET). The Virology Laboratory at Dr. R. Gutiérrez Children's Hospital is the Reference Laboratory for the National Measles Elimination Program of Buenos Aires City Hall (GCBA).

Ms. Barrero has a fellowship from the National Council of Investigation and Technology (CONICET) in the virology laboratory at the Dr Ricardo Gutiérrez Children's Hospital, Buenos Aires, Argentina. Her work is focused on molecular epidemiology of emerging and respiratory viruses.

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Cat or Dog Ownership and Seroprevalence of Ehrlichiosis, Q Fever, and Cat-Scratch Disease

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Concerns have been raised about the role of domestic cats or dogs in the acquisition of zoonoses, in particular in pregnant women or immune-suppressed persons. We report that cat or dog ownership is not associated with an increased seroprevalence of antibodies to *Anaplasma phagozytophilum*, *Coxiella burnetii*, and *Bartonella henselae* in symptom-free persons in Styria, Austria.

Keeping pet cats and dogs is very popular in Austria. However, these animals can serve as reservoirs for the agents of important bacterial infectious diseases and as a potential source of infection for humans, even though the infectious animals may be asymptomatic. Infections are potentially transmitted from domestic animals to humans by scratches, bites, or close contact. Examples for such infections include human granulocytic ehrlichiosis (*Anaplasma phagocytophilum*), cat-scratch disease (CSD, *Bartonella henselae*), and Q fever (*Coxiella burnetii*).

Cats are known to be the most important source of infections for *B. henselae* (aeroprevalence in Austrian cats is 33%) (1). However, dogs may also transmit *B. henselae* (2). Animals are contagious through their blood, which may contaminate saliva in cases of gum bleeding. Fleas from infected animals may contain the infectious agent, and bites from these fleas can transmit CSD. Typically, CSD is a benign and self-limiting disease in humans, occurring with lymphadenopathy, low-grade fever, primary cutaneous inoculation lesion, and weight loss, lasting 6–12 weeks. Rarely observed atypical signs and symptoms include erythema nodosum, figurate erythemas, thrombocytopenic purpura, Perinaud's oculoglandular syndrome, encephalopathy, hepatic granulomas, osteomyelitis, pulmonary disease, and optic neuritis (3). These severe manifestations occur in immunocompetent patients, whereas bacillary angiomatosis or peliosis hepatitis are more likely to develop in immunosuppressed patients.

C. burnetii infection has been associated with a chronic fatigue–like syndrome (4). Both cats and dogs are well-described reservoirs for *C. burnetii* (5). In humans, *C. burnetii* infection usually is asymptomatic (60%) or manifests as a mild disease with fever, headache, myalgias, and spontaneous recovery (5). However, this infection may lead to serious complications and even death in patients with acute disease, especially those with meningoencephalitis and myocarditis and, more frequently, in chronically infected patients with endocarditis. Q fever in pregnancy has been associated with abortion, premature birth, and low weight in newborns (6,7).

Within the past several decades, the number of *Ehrlichia* and *Anaplasma* spp. recognized to infect cats, dogs, and humans has expanded substantially (8). The agent of human granulocytic ehrlichiosis (HGE) has recently been classified as *A. phagozytophilum* (9). The disease has influenzalike symptoms with variable degrees of anemia, thrombocytopenia, leukopenia, and elevated liver enzymes. Dogs are thought to be sentinels for assessing risk for HGE in humans (10). Cases of HGE in the United States are increasing in incidence (11). Reports of acute cases in Europe have been rare, although serosurveys of the prevalence of antibodies to *A. phagocytophilum* have been conducted. A survey in Slovenia showed that 15.4% of the examined population had detectable antibodies to the pathogen and several cases of HGE had been confirmed (12). No similar serosurvey has been conducted in Austria, although *Ixodes ricinus*, thought to be the principal vector in Europe, is present in Austria (13). We report that the rate of seropositivity of *A. phagocytophilum* (immunoglobulin [Ig] G antibodies 25/376 [7%], IgM antibodies 6/376 [2%]), *B. henselae* (88/376 [23%]), and *C. burnetii* (23/376 [6%]) in Styria, Austria, is not affected by cat or dog ownership.

The Study

We examined serum specimens from 376 persons that were collected at the University Hospital Graz, Austria, from December 2001 to April 2002. These persons had no history of a tick bite for at least 1 year. A total of 202 persons with domestic dogs, cats, or both (dogs n=77, cats n=106, dogs and cats n=19) were compared with 174 persons who had no domestic pet contact for at least 1 year. Study participants in the no-pet group had never lived with a cat or a dog. The domestic pets had no symptoms or signs of infection, as determined by veterinarians. All participants were outpatients, and blood samples were drawn for routine blood tests. Each participant completed a questionnaire about medical history. These persons had no known history of rickettsiosis, borreliosis, or tick-borne

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encephalitis and reported no febrile or influenzalike illness during the previous 6 months. Each participant provided verbal consent for the serum to be used for detecting antibodies against several infectious agents related to zoonoses. The following information was collected for each participant: age, sex, area of residence, and medical history. Demographic data for the participants are shown in Table 1.

Serum samples were tested for the infectious agents with the following methods: *B. henselae* by indirect immunofluorescence (Biognost, Gräfelfing, Germany), *C. burnetii* by microagglutination reaction (Bodybion Mar; Bioveta National Enterprise Nitra, Slovakia), and HGE IgM indirect immunofluorescent antibody test (IFA, MRL Diagnostics, Cypress, CA), HGE by IFA IgG (Focus Technologies, Cypress, CA) with titers ≥64 were considered positive. All assays were performed in duplicate according to manufacturers' instructions. Biostatistical analysis was performed with the statistical package Jandel SigmaStat Statistical Software, version 2.0, NL (Jandel SigmaStat, Jandel, San Rafael, CA). Chi-square tests or t tests were used to determine differences between the presence of antibodies to the test organisms and demographic data, as appropriate. A two-tailed p value <0.05 was considered significant.

A total of 88 (23%) of 376 persons had antibodies against *B. henselae*. No differences in terms of age, sex, urban or rural residence, or concomitant diseases were noted. No difference in persons with and without domestic pets (46/174 [26%] and 42/202 [20%], respectively) was seen (Table 2).

The overall prevalence of antibodies to *C. burnetii* was 6% (23/376 persons). Again, no differences in age, sex, urban or rural residence, or concomitant diseases (13/202 [6%]) and persons with and without domestic pets (10/174 [6%]), were seen (Table 2).

The overall prevalence of IgG/IgM antibodies to *A. phagocytophilum* was 7% and 2% (25/376, and 6/376, respectively). No differences in terms of age, sex, urban or rural residence, or concomitant diseases were seen. IgG antibody titers were low (of 25 positive serum specimens, 17 had titers ≥64, and 8 had titers ≥128). In the six patients with positive IgM antibodies, no clinical evidence of ehrlichiosis was present, and additional blood samples showed no cytopenia. No difference in persons with (8% and 2% IgG/IgM positive, respectively) and without domestic pets (6% and 1% IgG/IgM positive, respectively) was seen (Table 2).

The seroreactivity to *C. burnetii* and *B. henselae* did not differ between *A. phagocytophilum*–positive patients and *A. phagocytophilum*–negative patients (p>0.05). Likewise, the seroreactivity to *A. phagocytophilum* and *B. henselae* did not differ between *C. burnetii*–positive patients and *C. burnetii*–negative patients (p>0.05). In addition, the seroreactivity to *A. phagocytophilum* and *C. burnetii* did not differ between *B. henselae*–positive patients and *B. henselae*–negative patients (p>0.05).

Conclusions

Veterinarians have the responsibility of providing accurate information to their clients about the zoonotic transmission of infections from pets, especially to those most vulnerable, such as children, pregnant women, the elderly, and the immunocompromised. Effective education is vital to allay public concerns and promote responsible pet ownership (14). With respect to *A. phagocytophilum*, *B. henselae*, and *C. burnetii,* cat or dog ownership was not related to an increased incidence of antibodies in our study.

CSD has been reported worldwide and seems to be the most common *B. henselae* infection in humans. In the United States, epidemiologic databases estimate that approximately 24,000 cases of CSD occur each year, with a calculated incidence of 9.3/10,000 ambulatory patients per year. In various studies, the seroprevalence of antibodies to *B. henselae* in humans ranges from 3.6% to 6% (15–17). Although CSD may occur in persons of any age, most patients are <18 years of age, perhaps because children are more likely to have close and rough contact with cats. The observed high incidence of antibody positivity in adults could be related to the persistence of antibodies after asymptomatic infection. Alternatively, it may be due chronic low-grade infection, which has been demonstrated for *B. quintana* and *B. bacilliformis* (18,19). The incidence of CSD is seasonal; most cases occur in August to October in northern temperate areas. The prevalence of the disease also varies by geographic location. The prevalence of antibodies to *B. henselae* is reportedly higher in areas with warm humid climates, where the prevalence and intensity of cat flea infestations are higher (20). Cats may infect humans either directly through scratches and bites or indirectly by means of the cat flea (*Ctenocephalides felis*), which is the arthropod vector (20). Recent research has demonstrated that *B. henselae* seroprevalence is elevated in patients with coronary vascular disease (21). Considering the comparatively high mean age of the patients tested in this study and the high percentage of

Table 1. Demographic data of patients with and without domestic pets

	No pet (n=174)	Cat (n=106)	Dog (n=77)	Cat and dog (n=19)
Age (y)	55±19	51±14	53±20	55±15
Sex (male/female)	55/119	32/74	37/38	6/13
Urban/rural	133/41	63/43	39/36	7/12

Table 2. Seroprevalence of antibodies against *Anaplasma phagocytophilum, Coxiella burnetti*, and *Bartonella henselae* in persons with and without domestic pets[a]

Organism	No pet n=174 (%)	Cat n=106 (%)	Dog n=77 (%)	Cat and dog n=19 (%)	No pet/cat p value	No pet/dog p value	No pet/cat and dog p value
A. phagocytophilum							
IgG[b]	12 (6)	7 (7)	2 (5)	4 (13)	0.239	0.332	0.162
IgM	2 (1)	1 (1)	1 (2)	2 (6)	0.856	0.849	0.611
C. burnetii	13 (6)	6 (6)	2 (5)	2 (6)	0.235	0.293	0.257
B. henselae	46 (26)	29 (28)	9 (23)	4 (13)	0.272	0.143	0.113

[a]No differences are evident by chi-square test for comparisons between the groups for all pathogens.
[b]Ig, immunoglobulin.

patients admitted for cardiovascular disease (Table 1), our results provide indirect support for this finding.

Because Q fever is rarely a notifiable disease, its incidence in humans cannot be assessed in most countries. Current epidemiologic studies indicate, however, that Q fever should be considered a public health problem in many countries, including France, United Kingdom, Italy, Spain, Germany, Israel, Greece, and Canada (Nova Scotia), as well as in many countries where Q fever is prevalent but unrecognized because of poor surveillance. Q fever remains primarily an occupational hazard in persons in contact with domestic animals such as cattle, sheep, and, less frequently, goats. Persons at risk from Q fever include farmers, veterinarians, abattoir workers, those in contact with dairy products, and laboratory personnel performing *C. burnetii* culture and working with *C. burnetii*–infected animals. However, reports of sporadic cases in persons living in urban areas after occasional contact with farm animals or after contact with infected pets such as dogs and cats have increased. Our data suggest that Q fever also occurs in Austria but that pet ownership or rural residence has no effect on seroprevalence. *C. burnetii* has also been isolated from the locally widespread *I. ricinus* ticks (22), although no tick-borne Q fever has been described in Austria.

For *A. phagocytophilum,* our seroprevalence is similar to those observed in Greece, much lower than that in Slovenia (15.4%), and higher than those seen in Bulgaria (2.9%) and Germany (1.9%) (23). In Austria (Styria), the prevalence in blood donors is 4% (24). We agree with Daniel et al. (23) that this Austrian prevalence could be due to the fact that the prevalence in blood donors is lower, unlike the survey in Greece, Slovenia, or this assessment. Even though the titers to *A. phagocytophilum* were low in our study, they suggest infection at an undetermined time.

Our data also suggest a discrepancy between the comparatively high seroprevalence of specific antibodies to *A. phagozytophilum*, *C. burnetii*, and *B. henselae*, respectively, and the few diagnosed cases of human disease. Clearly, cat or dog ownership is not related to an increased incidence of antibodies against these pathogens. However, because of the widespread distribution of *I. ricinus*, an organism also capable of transmission, this finding must be interpreted cautiously. In addition, the small sample size may have been insufficient to detect differences between the groups. Hence, recommendations for the population at risk for a severe course of these infections (pregnant women, the elderly, immunosuppressed patients, HIV-positive persons, and infants) are not possible. Nonetheless, such groups of patients may benefit from efficient tick and flea control on dogs and cats, as an adjunct to decrease mechanical transport of the parasites into their homes. Further research is needed to clarify the importance of pets or ticks for these diseases in Austria.

Dr. Skerget is a research fellow at the Department of Medicine, University of Graz. She is interested in zoonoses.

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Flying Squirrel–associated Typhus, United States

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In March 2002, typhus fever was diagnosed in two patients residing in West Virginia and Georgia. Both patients were hospitalized with severe febrile illnesses, and both had been recently exposed to or had physical contact with flying squirrels or flying squirrel nests. Laboratory results indicated *Rickettsia prowazekii* infection.

Typhus fever from *Rickettsia prowazekii* infection is a severe and occasionally fatal disease in humans. Frequently referred to as epidemic typhus or louse-borne typhus, this disease can cause large epidemics when conditions are favorable for person-to-person spread of body lice (*Pediculus humanus humanus*). For the last few decades, reported outbreaks have been confined mainly to the cold mountainous regions of Africa and South America and have disproportionately affected impoverished and displaced communities (1).

Infections with *R. prowazekii* are rarely described in the United States. From 1976 to 2001, a total of 39 human *R. prowazekii* infections were documented in persons with no reported contact with body lice or persons with lice (2–5). Nearly all of these cases were in the eastern United States, and in approximately one third of cases, contact with flying squirrels (*Glaucomys* spp.) or with flying squirrel nests occurred before disease onset.

Flying squirrels are the only known vertebrate reservoir of *R. prowazekii*, other than humans, and contact with these animals has been linked to most sporadic typhus cases in the United States. Interest in this disease was high in the 10 years after the first isolation of *R. prowazekii* from flying squirrels (6,7), but few cases have been reported since 1985. We describe two cases of flying squirrel–associated typhus that occurred in West Virginia and Georgia in 2002 and provide a contemporary summary of this disease in the United States.

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Case Reports

West Virginia

During February 2002, a 44-year-old man in West Virginia arrived in the emergency department, with headache, fever, and chills. The patient also had hematuria, joint pain, discomfort on the left side of his abdomen, and vomiting. Laboratory findings included elevated levels of alanine transaminase (ALT) and aspartate transaminase (AST) (100 and 91 U/L, respectively), leukocyte count of 4.1 x 10^9/L, platelet count of 249 x 10^9/L, and erythrocyte sedimentation rate of 42 mm/h. The patient also had diverticulosis. A treatment regimen of levofloxacin and metronidazole was begun, and the patient was admitted to the hospital. The condition worsened, and an infectious disease specialist was consulted on day 4 of hospitalization. At this time, the patient was febrile (maximum temperature, 38°C), reported myalgia and malaise, and had mildly injected sclerae (without photophobia). AST and ALT levels remained slightly elevated. Levofloxacin and metronidazole were discontinued. Because the patient was a recreational hunter, serologic tests for Rocky Mountain spotted fever, ehrlichiosis, adenovirus, Lyme disease, and cytomegalovirus (CMV) were ordered, and doxycycline was given as empiric therapy. Serologic tests were negative for all agents. The patient was discharged on day 7 of hospitalization with a diagnosis of immunoglobulin (Ig) A nephropathy and hepatitis. At a follow-up visit (day 27 after illness onset), the patient still had myalgias, fatigue, and conjunctivitis, although his fever and abdominal pain had resolved. At this time, serologic testing for typhus group rickettsiae showed reactive IgM antibodies at a titer of 512 and IgG at a titer of <64. An additional serum sample obtained on day 53 after illness onset was tested at the Centers for Disease Control and Prevention (CDC) by indirect-immunofluorescence assay (IFA) and showed titers of IgM and IgG antibodies reactive with *R. prowazekii* of 128 and 1,024, respectively, indicating a recent acute infection.

During January, the patient had spent several nights in a hunting cabin in a rural area of Hardy County, West Virginia. Flying squirrels had infested the cabin every winter for several years; evidence of nesting materials and rodent feces in the attic and wall spaces was visible. The patient did not report seeing flying squirrels during visits to the cabin in 2002 but reported having removed rodent nesting materials and debris from a wall space 10–15 days before becoming ill.

Georgia

During March 2002, a 57-year-old man from Fulton County, Georgia, received medical treatment for confusion associated with a febrile illness of approximately 1-week

duration. The illness was characterized by rigors, malaise, myalgia, headache, vomiting, anorexia, and cyclic fever. During medical evaluation, dehydration, atrial fibrillation, and abnormal results for liver enzyme tests were also found. Pronounced neurologic symptoms with expressive aphasia, impaired coordination, and confusion were demonstrated. The cerebrospinal fluid (CSF) sample had normal protein and glucose levels, was negative for bacteria by routine culture, and was negative for herpes simplex virus by polymerase chain reaction (PCR). Cefepime, ampicillin, and gentamicin were given, and a presumptive diagnosis of bacterial meningitis was made. Additional history was obtained from the patient's wife, who reported that 2 weeks before onset of symptoms, the patient had removed a flying squirrel carcass from the air intake chamber of the furnace in his office building's basement. He had also taken the furnace filter outside and brushed it vigorously to remove dust and animal hair that had collected over the winter. The infectious disease specialist prescribed doxycycline for treatment of suspected flying squirrel–associated typhus after a history of contact with these animals was established. The patient reported no history of having had a similar unexplained illness in the past and reported no contact with human body lice or with persons with lice.

Serum specimens obtained on days 7, 13, and 23 after illness onset were evaluated at CDC for antibodies reactive with *R. prowazekii* antigens. The titer of specific IgG antibodies was 8,192 for all three specimens. The patient was discharged from the hospital on day 9 and demonstrated normal mental status by day 10 of discharge.

Serum samples were collected from the eight people who also worked in the patient's office building. These specimens were screened by IFA for presence of antibody (IgG) reactive with typhus group rickettsiae antigens. All were negative (titer <16).

Cases from 1985 to 2002

A review of records at CDC identified two additional cases of flying squirrel–associated typhus during 1985 to 2002 (Table). Typhus is not a nationally notifiable disease in the United States, and public health officials become aware of cases only when specialized confirmatory laboratory assays, performed at state health departments or CDC, are requested.

Conclusions

Inhalation and transdermal or mucous membrane inoculation of infected louse feces are well-established routes of pathogen transmission during epidemics of human louse-borne typhus. The mechanism by which *R. prowazekii* is transmitted from flying squirrels to humans is less well understood. Various routes have been hypothesized, but none have been empirically established. Plausible mechanisms include inhalation or direct introduction (through mucous membrane or dermal abrasion) of infected feces from louse or flea ectoparasites of flying squirrels or through the bite of infected flea ectoparasites of flying squirrels (9). At least one species of flea ectoparasite (*Orchopeas howardii*) of flying squirrels is known to opportunistically bite humans and could serve as a bridge

Table. Epidemiologic and clinical characteristics of flying squirrel–associated typhus fever in the United States, 1984–2002[a]

	Case no.			
Characteristic	I	II[b]	III	IV
Sex	Male	Female	Male	Male
Age (y)	54	54	44	57
State of residence	Massachusetts	North Carolina	West Virginia	Georgia
Mo of onset	February 1998	September 1999	January 2002	March 2002
Flying squirrel contact	Y	Y	Y	Y
Serologic titer (no. onset)				
IgG *Rickettsia prowazekii*	4,096 (10)			8,192 (7)
				8,192 (13)
	32,768 (38)	nd	128 (53)	8,192 (23)
IgG *R. typhi*	nd	nd	<32 (53)	4,096 (7)
				4,096 (13)
				4,096 (23)
IgM *R. prowazekii*	nd	2,048 (18)	1,024 (53)	nd
IgM *R. typhi*	nd	512 (18)	1,024 (53)	nd
Symptoms				
Max fever (°C)	nr	40°C	38°C	40°C
Chills	Y	nr	Y	Y
Headache	Y	Y	Y	Y
Rash (type, location)	nr	Y (macular, trunk)	N	Y[c]

[a]Ig, immunoglobulin; Y, yes; N, no; nd, not done; nr, not recorded.
[b]Recurrent illness, first episode January 1999, flying squirrel contact, rash at that time. Other symptoms associated with both episodes. Serologic titer from second episode.
[c]Rash herpetic, not directly attributable to *R. prowazekii* infection but commonly seen in context of classic louse-borne epidemic typhus (8).

vector for transmission from flying squirrel to human. Rickettsiae transmission among captive flying squirrels, however, has only been demonstrated with a louse vector (9,10). While the exact mechanism of pathogen transmission has not yet been determined, the lack of detectable exposure to *R. prowazekii* in the household members or coworkers of documented cases (3,5) supports the idea that the risk for *R. prowazekii* infection after casual or indirect exposure to flying squirrels is low. Rather, existing evidence suggests that infection follows from close physical contact with flying squirrels or from exposure to a concentrated source of infectious materials (e.g., nests, dander, or infected ectoparasites).

Currently, no formal system for epidemic typhus surveillance exists in the United States, and diagnosis is hindered by the lack of rapid and reliable commercial tests. *R. prowazekii* infections can be confirmed by serologic testing, PCR, or organism culture. Commercial testing is, however, not widely available, and commercial serologic tests lack specificity because most detect antibodies reactive with a surrogate typhus-group rickettsial antigen (typically *R. typhi*). PCR (in conjunction with DNA sequencing) is a highly specific diagnostic tool (11) but has low sensitivity for commonly available clinical specimens, such as whole blood or serum. PCR may be used to greater effect on other clinical specimens (e.g., cerebrospinal fluid, lymphocytes, and skin biopsy) if they are collected and submitted for testing. Specific serologic and molecular testing is available at CDC for specimens submitted through state public health laboratories.

Sporadic epidemic typhus occurs in the United States, primarily during winter and spring, and in regions within the normal range of the southern flying squirrel (*Glaucomys volans*) (12). This illness can be severe, resulting in protracted hospital stays, particularly when diagnosis and appropriate treatment are delayed. In these two cases, treatment with tetracycline antibiotics was initiated after other broad-spectrum antibiotics were used. When therapy was changed to the appropriate antibiotic, the response was rapid. Tetracycline antibiotics are highly effective therapies for typhus (13). These cases underscore the importance of obtaining a thorough history of animal and arthropod contact in patients with acute febrile illness. Physicians and healthcare providers should remain alert to the signs and symptoms of epidemic typhus and be aware of appropriate diagnostic methods and antibiotic treatments (13,14).

Dr. Reynolds has a Ph.D. in biomedical science and has pursued postdoctoral research in evolutionary biology. She is a fellow in the Epidemic Intelligence Service at the Centers for Disease Control and Prevention in the Viral and Rickettsial Zoonoses Branch, Division of Viral and Rickettsial Zoonoses, National Center for Infectious Diseases.

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Chlamydia trachomatis Infections in Female Soldiers, Israel

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We examined the prevalence of *Chlamydia trachomatis* infection in Israeli female soldiers. The prevalence was 3.2% among soldiers seeking medical care; rural residence was identified as a significant risk factor. Nevertheless, given the study design, recommending broad-scale screening of Israeli female soldiers may be premature.

Recent studies from the United States and Europe report that the prevalence of *Chlamydia trachomatis* ranges from 5% to 20% in sexually active persons (1,2). Among women, the consequences of the disease include pelvic inflammatory disease, ectopic pregnancy, and infertility, sequelae often accompanied by a substantial economic impact (3). Most importantly, for up to 80% of infected women, infection is asymptomatic, resulting in failure to seek timely medical care and the exacerbation of such sequelae (4). Consequently, screening programs have been recommended to reduce infection, transmission, and disease consequences (4,5). However, before such programs are universally advocated, since the cost-effectiveness of such control programs is at least partially contingent upon disease prevalence (5), more data are required to assess the extent to which such prevalence rates may indeed be generalizable to other countries and populations.

Epidemiologic research indicates that prevalence rates of *C. trachomatis* vary, with a number of risk factors accounting for a substantial portion of this variance (4). For example, in the landmark study by Gaydos et al., significant risk factors for the prevalence of this infection among American female military recruits included age (women <25 years of age were at higher risk), vaginal intercourse, lack of condom use, and multiple sexual partners (4). Given that sexual practices such as condom use and multiple sex partners are likely to vary from country to country, we designed a study to assess the prevalence of *C. trachomatis* in a population of Israeli women, namely female soldiers, who, according to previous research, had a heightened risk of having the disease because of their age and sexual activity (4–6).

The Study

Whereas Gaydos et al. invited all female army recruits undergoing a physical examination at a reception base over the course of 2-year period to participate in their study, our sample consisted of Israeli women actively seeking medical care. Specifically, participants in the current study were women serving in the Israel Defense Forces, who, over a 12-month period attended a women's health clinic for a routine gynecologic examination, treatment of genitourinary symptoms, or contraceptive counseling. The clinic was associated with a major tertiary hospital in northern Israel. Of the 800 women we asked, 708 volunteered to participate, for a response rate of 88.5%. Participants were between the ages of 18 and 45 (median age 19, mean age 20). Most participants (77%) were born in Israel.

After signing an informed consent form, participants were asked to complete a short questionnaire (including demographic and sexual history items) and provide a urine specimen. We performed the polymerase chain reaction (PCR) of urine specimens to test for *C. trachomatis* and *Neisseria gonorrhoeae* (Roche Amplicor, Branchburg, NJ). The PCR results and questionnaire data were coded as dichotomous variables and examined on the basis of both univariate (chi-square and Fisher exact test) and multivariate (logistic regression) analysis.

Of the 708 participants, 23 (3.25%) tested positive for *C. trachomatis,* and 8 (1%) tested positive for *N. gonorrhoeae.* These rates are substantially lower than the 9.2% prevalence rate for *C. trachomatis* reported by Gaydos in the U. S. military study (4). The median and average age of those affected was 20. Most of those testing positive reported 1) having had sex in the past 3 months (86%), 2) having had more than one partner (45%) or having had a partner who had had more than one partner (57%), or 3) never having used a condom (70%) (Table). Nevertheless, none of these factors, identified by Gaydos as significant risk factors in the American sample, was found to be a significant predictor of *C. trachomatis* infection in the Israeli soldiers. Similarly, while most of those testing positive were born in Israel (81%), country of origin was not a significant predictor of infection. Neither were gynecologic symptoms found to significantly distinguish between *C. trachomatis*–positive and –negative soldiers. Indeed, the only factor that we found to significantly distinguish

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Table. Risk factors for *Chlamydia trachomatis* in female Israeli soldiers

Risk factor	Presence in soldiers who were		p value
	Positive for *C. trachomatis*/no. tested (%)	Negative for *C. trachomatis*/no. tested (%)	
Sex in the past 3 months	19/22 (86)	585/629 (93)	0.999
>1 partner	9/20 (45)	241/636 (38)	0.519
Partner having >1 partner	8/14 (57)	181/518 (35)	0.093
Never used a condom	14/20 (70)	442/628 (70)	0.99
Country of origin			0.95
Israel	17/22 (81)	525/685 (77)	
Other	5/22 (19)	160/685 (23)	
Dysuria	4/21 (19)	136/639 (21)	0.805
Vaginal discharge	11/21 (52)	263/635 (41)	0.316
Location of residence			0.049
Urban	5/21 (24)	310/682 (45)	
Rural	16/21 (76)	372/682 (55)	

between *Chlamydia*-positive and -negative persons was urban versus rural residence. Specifically, rural residents were significantly more likely to be positive for *C. trachomatis* than urban residents (chi square=3.86, $p<0.05$). Similarly, in a logistic regression analysis, only rural residence was significantly associated with *Chlamydia* infection ($p=0.043$) (Table).

Conclusions

The relatively low rate of *C. trachomatis* prevalence was somewhat surprising for several reasons. First, since 1994, when *Chlamydia* infection became a nationally notifiable infectious disease in Israel, the number of cases reported annually has risen 10-fold. Secondly, since 2000, 14% of symptomatic soldiers attending the sexually transmitted disease clinic of another major medical center in northern Israel were diagnosed with *Chlamydia* infection. Finally, while Gaydos et al. found a 9.2% prevalence rate in a sample of soldiers reporting for a general physical examination, our sampling strategy focused on women who had a high probability of being sexually active (i.e., seeking contraceptives) and consequently, according to the previous literature, were at higher risk of contracting asymptomatic *C. trachomatis* infection (5).

However, oversampling those most concerned with their health (i.e., "the worried well") may partially explain the lower than expected prevalence rate. Indeed, given the risk for a self-selection bias inherent in such a sampling strategy, one important limitation of the current study is that the sample population may not be entirely representative of the entire population of female soldiers in the Israeli Defense Forces. A second limitation is that the Roche PCR kit used in this study was not cleared by the Food and Drug Administration for testing female urine samples for *N. gonorrhoeae* because of its low sensitivity. Consequently, the actual prevalence of gonorrhea may in fact be higher than reported above.

Given the nearly universal draft of young Jewish females in Israel, however, our results may provide a strong indication of the prevalence of *C. trachomatis* in young, sexually active Jewish women in that country. Despite the relatively low rate of prevalence, based on a number of cost-efficacy studies (6,7) and consistent with the recommendations of the Centers for Disease Control and Prevention (5), focused screening of asymptomatic, sexually active female soldiers, by virtue of their age, may be a cost-effective mode for identifying those with *C. trachomatis* infection in Israel. Identifying gonorrhea in 1% of the women tested shows an important secondary benefit to such screening. Nevertheless, before recommending the adoption of a broader, more focused screening program of female soldiers, whether symptomatic or not, we recommend collecting additional data with a more randomized sampling strategy similar to that adopted by Gaydos et al. (4). Were an equivalent or higher prevalence rate to be found in a random sample of female soldiers during their service, then, according to recent estimates of cost-effectiveness (7,8), such a screening program would be worthy of consideration.

Acknowledgments

We thank Ada Tamir for her assistance with the data analysis.

This research was partially funded by the Israel Defense Forces (Grant # 15000615-01).

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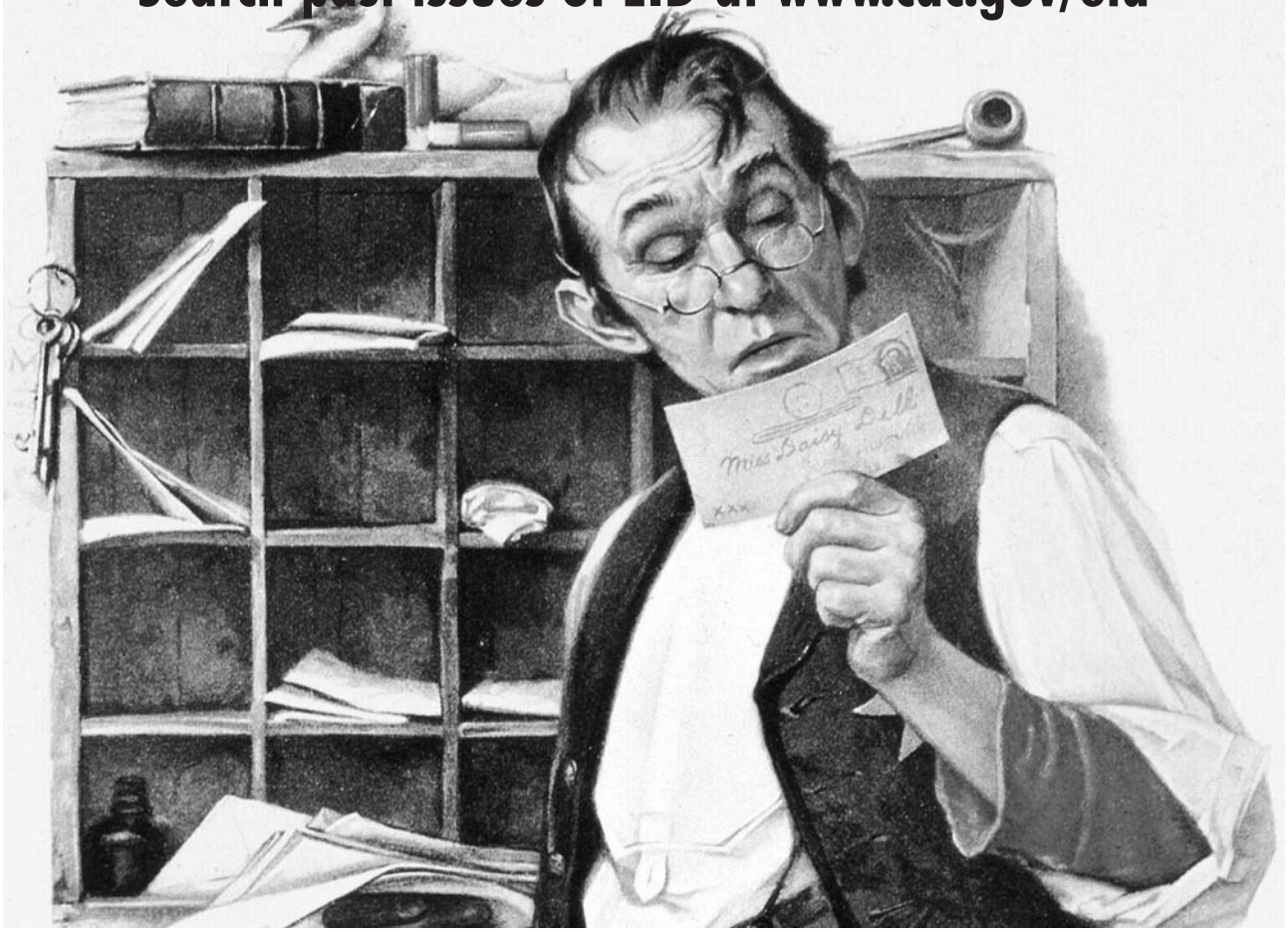

Clostridium tertium in Necrotizing Fasciitis and Gangrene

To the Editor: Bacterial species of the genus *Clostridium* are anaerobic or aerotolerant, gram-positive, endospore-forming bacilli found in the soil and gut of humans and other animals. These species cause botulism, tetanus, gas gangrene, antibiotic-associated diarrhea, pseudomembranous colitis, foodborne diarrhea, and necrotic enteritis in humans and infections in other animals. *Clostridium tertium*, a non–exotoxin-producing, aerotolerant species, is an uncommon human pathogen. First isolated by Henry from war wounds in 1917 (1), *C. tertium* was recognized as a human pathogen when cases of bacteremia were reported in 1963 (2). The organism has been implicated in bacteremia (3,4), meningitis (5), septic arthritis (6), enterocolitis (7), spontaneous bacterial peritonitis (8), posttraumatic brain abscess (9), and pneumonia (4). Miller and colleagues, in a recent review of 32 cases, highlighted the role of neutropenia, intestinal mucosal injury, and exposure to β-lactam antibiotics predisposing to *C. tertium* bacteremia (3). *C. tertium* as the sole pathogen causing necrotizing fasciitis and gangrene has not been reported. We report the first two cases of necrotizing fasciitis and gangrene caused by *C. tertium*.

A 58-year-old man was seen at the Postgraduate Institute of Medical Education and Research, Chandigarh, India, on August 17, 2001, with two nonhealing, punched-out ulcers near the right lateral malleolus. The ulcers were approximately 2 cm x 2.5 cm in size, with necrotic margins, purulent exudate, and a foul odor. On the second day, temperature of 39.4°C and gangrene of the right leg developed. The patient also had alcoholic liver disease and non-Hodgkin lymphoma, for which he had been receiving chemotherapy for last 6 months, and he had had a 6 months' course of antitubercular combination therapy for pulmonary tuberculosis 2 years earlier.

Peripheral blood showed a leukocyte count of 10,000/mm^3 with 80% neutrophils, 11% lymphocytes, and 9% monocytes. The fasting blood glucose level was 400 mg/dL with normal electrolytes and renal function test results. Liver function tests showed a serum glutamic oxalacetic transaminase level of 80 IU/L and a serum glutamic pyruvic transaminase level of 75 IU/L. Sputum microscopy showed no acid-fast bacilli. Microscopy of necrotic tissue showed gram-positive bacilli (1–1.5 μm x 5–6 μm) with frequent oval terminal spores. Aerobic blood culture was sterile. Insulin was given to control his blood glucose level. Antimicrobial therapy included intravenous metronidazole, vancomycin, and imipenem. Skin and subcutaneous tissue debridement and fasciotomy were also undertaken.

A 40-year-old man was seen at the Postgraduate Institute on October 17, 2001, with multiple injuries of lower extremities and abdomen following a motor vehicle accident. After 24 h, the patient's left leg and thigh turned gangrenous, and a high-grade fever (38.6°C) and an elevated leukocyte count of 14,000/mm^3 (70% neutrophils, 20% lymphocytes, 10% monocytes) developed. Microscopy of necrotic tissue showed gram-positive rods (1 μm x 5 μm) with oval, terminal spores. Aerobic blood culture was sterile. Skin and subcutaneous tissue were extensively debrided. Antibiotic therapy with intravenous penicillin, metronidazole, and amikacin was instituted.

Necrotic tissues from both cases were cultured on Columbia sheep blood agar plates incubated aerobically and anaerobically (ANOXOMAT system, MART Microbiology BV, Lictenvoorde, the Netherlands) at 37°C for 24 h and 48 h, respectively. Overnight aerobic culture grew small gray colonies (<1 mm in diameter) of non–spore-forming gram-variable bacilli (1 μm x 5 μm), which, on subculture anaerobically grew gram-positive rods with oval, terminal spores. Anaerobic culture directly from specimen yielded similar spore-forming, gram-positive bacilli. The isolates were presumptively identified as *Clostridium* species by colony characteristics, Gram-stain morphology, and negative catalase test results; they were confirmed as *C. tertium* based on aerotolerance; shape and location of endospores; fermentation of glucose, lactose, maltose, and sucrose; nitrate reduction; and absence of proteolysis. Both isolates of *C. tertium* were susceptible in vitro to penicillin, ampicillin, vancomycin, and metronidazole.

C. tertium has been traditionally considered nonpathogenic. The organism was earlier isolated along with pathogens such as *C. perfringens*, *C. septicum,* and *C. sordellii* from war wounds and cases of gangrene (1). *C. tertium* is being increasingly reported as a human pathogen (3–9), and the strongest association has been with septicemia in patients with neutropenia and hematologic malignancies (3,4). Predisposing factors for *C. tertium* bacteremia include intestinal mucosal injury, neutropenia, β-lactam antibiotics (third-generation cephalosporins), cytotoxic chemotherapy, and severe liver disease, as reviewed by Miller and co-workers (3). Necrotizing fasciitis and gas gangrene caused by *C. tertium* as the sole pathogen have not been reported, although Miller reported necrotizing fasciitis in a patient with acute lymphocytic leukemia with *C. tertium* and *C. septicum* isolated from blood (3). The importance of isolation of *C. tertium*, particularly in polymicrobial cultures, is not well-established. In our report, the first patient suffered from alcoholic liver disease, had very

high blood glucose levels, and was on cytotoxic chemotherapy for previous 6 months. The risk for intestinal injury is high in severe liver disease and cytotoxic chemotherapy. Intestinal mucosal compromise may potentiate translocation of *C. tertium* to systemic circulation and metastatic foci. The second patient had no predisposing medical history before the present episode that might have resulted in acquisition of *C. tertium* from the soil. Both patients had pyrexia, necrotizing fasciitis, and gangrene of a lower limb with *C. tertium* as the sole bacterial isolate. Neither patient had neutropenia when they were first seen. This contrasts with earlier reports of *C. tertium* infections (predominantly bacteremia), which usually occurred in patients with preexisting neutropenia (3). Both patients improved with penicillin or vancomycin and metronidazole, and both isolates were susceptible to these three antibiotics in vitro. Therefore, we consider both isolates to be clinically important. The pathogenesis of infection caused by *C. tertium* is not well understood, since the organism does not produce exotoxins. No evidence exists to correlate oxygen sensitivity with bacterial enzyme production and pathogenicity in aerotolerant clostridia. Our report adds to the list of recently emerging diseases caused by *C. tertium*. The growing acceptance of this organism as a human pathogen will lead to better delineation and understanding of its pathogenic potential.

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Dengue Hemorrhagic Fever, Uttaradit, Thailand

To the Editor: Dengue hemorrhagic fever (DHF) has been recognized as a disease of young children in the past. Three decades ago most reported case-patients in Thailand were 3–6 years of age (1). Increasing evidence shows that the age group most affected is changing (2). We report evidence that in Uttaradit, Thailand, the predominant age of those who acquire DHF has increased by at least 2 years during the 1990s.

Uttaradit is a province in the northern part of Thailand. DHF is endemic in Uttaradit, as it is in most parts of the country. Between 1992 and 2001, three major outbreaks of DHF occurred, in 1993, 1998, and 2001.

The number of DHF cases reported to the Provincial Health Office from January 1992 to December 2001 (classified by age groups) was used as the estimated annual DHF incidence. Case definition and categorization followed the International Statistical Classification of Diseases and Related Health Problems (ICD-10). DHF categories reported in this study included both DHF without shock and the dengue shock syndrome (the number of cases and deaths combined). Dengue fever, a milder disease manifestation, was not included.

The age distribution of DHF cases showed that, in the 1993 epidemic, children 5–9 years of age had the largest proportion of cases, whereas in 2001, the peak age of those infected was 10–14 years. The transitional stage (mean age 11.3 years) was observed in 1998.

During the observed period, the annual mean age of DHF case-patients ranged from 8.4 to 15.1 years. Despite some fluctuation, the mean age of DHF case-patients was <10 years of age before 1996. From 1997 onward, the mean age was consistently >10 years.

The incidence of DHF in children ≤4 years of age decreased from 586.0/100,000 in the 1993 epidemic to 197.5/100,000 in 2001. The incidence in children 5–9 years of age also decreased from 1,330.3/100,000 to 676.6/100,000 in the corresponding years. While the incidence in children 10–14 years of age remained unchanged, the incidence in those 15–24 years of age increased from 122.8/100,000 to 323.5/100,000, and from 20.0 to 52.6 per 100,000, a more than twofold increase.

Our results clearly showed that the mean age of DHF case-patients in-

creased from 10.0 years in the 1993 epidemic to 11.3 years in 1998 and to 13.2 years in 2001, as a consequence of a decrease in the incidence among children ≤9 years, and an increase in the incidence among the older age groups. This finding was similar to what had been observed earlier in Singapore and Indonesia (2,3).

Some researchers have found that when the average number of annual dengue infections declines, the chance of persons acquiring dengue infections declines, resulting in delays in the age when a person has experienced the first, then second, dengue infection (4). However, in Uttaradit, as well as in other parts of Thailand, dengue infection is endemic, with large outbreaks occurring at 2- to 3-year intervals: later epidemics have also shown an increase in the overall incidence rates. Thus, this explanation is unlikely to be the reason for a shift in the age distribution of DHF in Uttaradit.

We reviewed information that indicated that the shift in age predominance could be caused by the changes in places of transmission. Among these was the study in Singapore, which proposed that an effective mosquito-control program in households had resulted in changes in which age group had the largest number of DHF cases (5). A significant ($p<0.001$) rise in seroconversion in children ≥6 years of age coincided with the start of formal schooling. The likelihood of dengue infection increased with time spent away from home, suggesting that the location where dengue was acquired may have changed (5). The recent study in Thailand also suggested that, although dengue infection may be transmitted in the home environment, transmission within schools may also be important (6).

The changing of the population age structure also explained the age shifting phenomenon in some studies (7). In Uttaradit, however, changes in the age structure of the population were small from 1992 to 2001.

The intervening effect of vaccination against Japanese encephalitis virus, a different but related flavivirus, could also explain why the mean age for most cases of DHF increased. Cross-reaction between dengue virus and Japanese encephalitis virus is well established (8). Vaccination against Japanese encephalitis virus may temporarily protect persons, primarily young children, against dengue infection or at least reduce its severity, resulting in a decline in the observed incidence. The cohort of these vaccine recipients were then exposed to dengue infection later in life and exhibited diseases when they shifted into an older age group. An increase of Japanese encephalitis vaccine coverage from 96% in 1995 to 100% in 2001 (9) appeared to confirm the above explanation. Nevertheless, areas where Japanese encephalitis vaccination had not been implemented also experienced a change in the age group with the most DHF. A final alternative explanation is the effect of herd immunity. Some researchers have observed that in places where dengue does not occur yearly, older age groups have higher rates of infection (10). However, dengue cases had been reported every year in Uttaradit, and the intervals between each epidemic were not long. We therefore, believed that the herd immunity hypothesis did not explain the observed changing age predominance in our study.

The mean age of DHF case-patients in Uttaradit, Thailand, increased by ≥3 years between 1992 and 2001. This phenomenon may be important from a public health standpoint, as community and health-related personnel may still perceive DHF as a disease of only small children and unintentionally leave older children less protected or ignored. Further study is needed to confirm that the age group shifting of DHF predominance can be explained by the changes in locations where disease transmission takes place and possibly by effective household mosquito- elimination programs.

Acknowledgments

We thank the staff of Uttaradit Provincial Health Office for their contribution on dengue reports.

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Antimicrobial Drug–resistant *Salmonella* Typhimurium (Reply to Helms)

In Reply to Helms: In the article by Helms et al., Helms concludes that infections with *Salmonella* Typhimurium strains resistant to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline (hereafter referred to as penta-resistant) were associated with higher death rates than infections with non–penta-resistant *S.* Typhimurium. Helms also concluded that infections with quinolone-resistant (nalidixine-resistant) *S.* Typhimurium were associated with higher death rates than quinolone-susceptible *S.* Typhimurium (1).

Table 2 in Helms' article provides information that enables close scrutiny of this conclusion and comparison of the excess mortality associated with penta-resistant, quinolone-susceptible *S.* Typhimurium with the excess mortality of non–penta-resistant *S.* Typhimurium (1). In this letter, the Table is based on the original table. However, two additional comparisons have been added: the p values, which are not based on the data but are approximations based on the parameters in the table.

The conclusion is that only quinolone resistance is associated with excess mortality compared with nonresistant isolates. Penta-resistant, quinolone-susceptible *S.* Typhimurium has a risk ratio of 2.9 (1.1 to 7.9) compared to the ratio of non–penta-resistant isolates 2.1 (1.5 to 2.9). When these figures are compared, the approximate p value is 0.55, which, of course, is far from being significant. Thus, on the basis of the article by Helms, penta resistance may not pose a greater threat to human health than non–penta resistance. However, the measured effect of penta resistance is achieved by the inclusion of quinolone-resistant *S.* Typhimurium in the group.

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Table. Table showing additional comparisons (1)[a]

	Resistant		Susceptible		
	Deaths/ cases	RR[b] (95% CI)	Deaths/ cases	RR[b] (95 % CI)	p value
Penta with and without quinolone	12/283	4.8 (2.2 to 10.5)	47/1,764	2.1 (1.5 to 2.9)	0.06
Penta with quinolone	5/40	13.1 (3.3 to 51.9)	47/1,764	2.1 (1.5 to 2.9)[c]	0.01[d]
Penta without quinolone	7/243	2.9 (1.1 to 7.9)	47/1764	2.1 (1.5 to 2.9)[c]	0.55c[d]

[a]RR, relative risk; CI, confidence interval.
[b]Adjusted for coexisting conditions.
[c]Compared to the non-penta group.
[d]Approximations based on the parameters from the table.

Antimicrobial Drug–resistant *Salmonella* Typhimurium (Reply to Dahl)

In Reply to Dahl: The emergence and spread of multidrug-resistant *Salmonella enterica* serovar Typhimurium DT104 (MDR DT104) contributed to an international increase in antimicrobial drug resistance in *S.* Typhimurium in the late 1990s (1,2). This type of *Salmonella* is usually resistant to five drugs: ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (R-type ACSSuT) and easily acquires resistance to other drugs, including quinolones, trimethoprim, and aminoglycosides (1,3–5). To determine death rates after infection with MDR DT104 or closely related strains, we identified patients who were infected with strains at least resistant to ACSSuT (6). Analysis limited to strains that were only R-type ACSSuT would have given a misleading result since MDR DT104 often, as mentioned, develops additional resistance to other classes of antimicrobial drugs in addition to the ACSSuT-complex. This fact needs to be taken into account in any attempt to quantify the overall public health impact of MDR DT104 and related strains.

We found, in our matched cohort study (6), that 283 patients infected with strains resistant to at least ACSSuT were 4.8 times more likely to die than the general Danish population, compared with 2.3 for 953 patients infected with pansusceptible strains. This difference in death rates ocurred mainly because 40 of the 283 strains had R-type ACSSuTNx (i.e., additional resistance to nalidixic acid), and infection with this strain in particular is associated with a high death rate (relative mortality 13.1). As Dahl suggests, infection with R-type ACSSuT (Nx susceptible) was not

associated with excess mortality in the 243 patients included in the analysis, and the measured effect of ACSSuT was achieved by the inclusion of the nalidixic acid–resistant strains in this group. However, all deaths associated with nalidixic acid–resistant strains occurred in the 40 patients with R-type ACSSuTNx (being DT104s), whereas none of the 43 patients infected with non-ACSSuT strains resistant to nalidixic acid died. This finding may be related to small numbers in these subanalyses. However, because 25 of the patients with R-type ACSSuTNx were part of an outbreak, they may have had an average higher exposure dose, which may have contributed to some deaths (3). In addition, an interaction between different resistance traits in Salmonella may exist, which may lead to more deaths and disease, or DT104 may be somewhat more virulent than most other *S.* Typhimurium subtypes.

The database that we used for our analysis was updated in May 2002. We have now identified 13 deaths in 342 patients infected with strains resistant to ACSSuT (but Nx susceptible), which corresponds to a relative mortality rate of 4.18 (95% confidence interval [CI]) 2.18 to 8.02) compared with a matched sample of the general population. Of 1,432 patients infected with pansusceptible strains, 43 patients died (relative mortality rate 2.64; 95% CI 1.88 to 3.70). In other words, the mortality rate in patients infected with strains resistant to ACSSuT (Nx susceptible) was 1.6 times higher than in patients with pansusceptible strains (p value for homogeneity 0.22). These estimates were not adjusted for coexisting conditions as were the estimates in the paper (6).

We agree with Dahl that particular problems are associated with quinolone resistance in zoonotic salmonellae and that fluoroquinolones may have reduced efficacy to treat patients infected with *Salmonella* strains that are nalidixic acid (quinolone) resistant (7). We therefore encourage initiatives to preserve the efficacy of fluoroquinolones, including a limitation of their use in agriculture. Whether infection with *S.* Typhimurium R-type ACSSuT, with no additional resistance, is associated with higher disease or death rates than pansusceptible *S.* Typhimurium remains unclear. Although the difference was not significant (p=0.22), our recent estimates suggest that the death rate is approximately 60% higher in patients infected with such strains. This view is corroborated by recent studies from the United States, which suggest that *S.* Typhimurium R-type ACSSuT is associated with an increased risk for blood stream infection (8) and that resistance in nontyphoid *Salmonella* is associated with an increased risk for admission to hospital (9).

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Serogroup A *Neisseria meningitidis* Outside Meningitis Belt in Southwest Cameroon

To the Editor: Epidemic meningitis associated with serogroup A *Neisseria meningitidis* is a devastating disease in the absence of vaccination (1). Without treatment, the case-fatality rate is high, approaching 100%. In Africa, such epidemics occur regularly (1) within a well-limited geographic zone, the so-called

African meningitis belt (2). In the countries within the meningitis belt, the illness is endemic and sporadic: numerous cases of meningococcal meningitis are reported each year during the dry season, and every 6–12 years a large outbreak occurs. Serogroup A *N. meningitidis* also causes sporadic cases of meningitis outside the meningitis belt, accounting for 10% to 30% of cases with identified causes (1,3). Outbreaks may also occur outside of the belt, but they do not exhibit the same epidemiologic aspects. We report an epidemic of meningococcal meningitis in the South-West Province of Cameroon (~500 km south of the African meningitis belt and 350 km east of Yaoundé, the country's capital), involving 61 cases and 13 (21%) deaths.

Clinical and epidemiologic information was collected from medical records at Bechati Health Centre and Fontem Missionary Hospital, the only two care centers in the epidemic area. A case was defined as sudden fever ≥38°C and neck stiffness if the patient was >12 months of age, or bulging fontanelle if the patient was <12 months of age. Other symptoms of meningitis, such as nausea, vomiting, irritability, confusion, and lethargy to the point of coma, were observed in several patients. An epidemic threshold of 15 cases reported in a 2-week period in a population of >100,000 has the specificity and probability to predict a meningococcal epidemic within the meningitis belt (1). In the Bechati area (10,326 inhabitants), nine fatal cases occurred during the first week (March 5–12, 2000), for an attack rate of 87 per 100,000, a figure that local health authorities considered as epidemic.

The outbreak extended from March 6 to April 6, 2000, and peaked March 21–22 (10 cases in 48 hours), in the area of Bechati (5°40′ north of the equator, 300 km^2). The first case-patient was retrospectively identified as a 9-month-old child from Bechati, treated for meningitis at Fontem Missionary Hospital on February 25. This child was shown to have a gram-negative *Diplococcus* infection by microscopic analysis of a cerebrospinal fluid (CSF) sample. (Fontem is a rural city, 15 km from Bechati, by a poor-quality railroad track.) The epidemic began on March 5 in Bechati and spread to seven other villages. The last case was recorded on April 6. After the index case, 6 cases occurred in week 1; 16 cases in weeks 2, 3, and 4; and 6 cases in week 5. A total of 61 cases were registered in the Bechati Health District (33 male and 28 female patients), with a mean age of 22 years (range 9 months to 70 years) and an attack rate of 591 per 100,000 (61/10,326).

The first patient was cured by appropriate treatment with thiamphenicol at Fontem Missionary Hospital. The next nine case-patients in these remote villages all died, either without treatment or despite traditional treatment. As this meningococcal epidemic was the first of its kind in this area, introducing an efficient response took some time. The public health authorities introduced chloramphenicol treatment on March 13; subsequently, four more deaths occurred, including two untreated patients, among the next 51 case-patients. The death rate was 100% in week 1 (6/6), 31.3% in week 2 (5/16), 0% in week 3, 12.5% in week 4 (2/16), and 0% in week 5. Deaths were more frequent in patients >20 years of age (12/34; 35%) than in younger patients (1/27; 4%, $p<0.01$), and deaths were highest in 20- to 29-year-old patients (8/17; 47%). These findings suggest that the adults were affected earlier than children and teenagers. Two (4%) of the 50 patients treated with chloramphenicol died, whereas all 11 (100%) untreated patients died.

Nine of the 61 patients underwent some sort of CSF analysis. One had a positive direct microscopic examination. Four of the eight CSF samples taken in the field tested positive for meningococcus A in the rapid agglutination test; from one of these samples, serogroup A *N. meningitidis* was isolated. Thus, we considered that the epidemic was due to meningococcus A. The strain isolated was susceptible to major antibiotics, resistant to trimethoprim/sulfamethoxazole, and belonged to the epidemic clone A:4:P1.9 (sequence type ST-7), which was circulating simultaneously in north Cameroon and south Chad. The third pandemic caused by this strain began in China in 1993, causing large epidemics in Mongolia in 1994 and Moscow in 1996 (4). This sequence type seemed to emerge in Africa since 1995 (5), and researchers hypothesized that severe epidemics attributable to this ST-7 clone occurred in Cameroon and Niger, since such strains were circulating in the population and this ST-7 clone was responsible for severe outbreaks in Chad (1998) and Sudan (1999) (5). After meningococcus A was identified on March 22, the decision was made to vaccinate the local population. Polysaccharide A-C meningococcal vaccines were administered soon after week 6 in the outbreak area (i.e., after the epidemic ended).

Bechati is located in an area of tropical rainforest, with mountains at an altitude of ~1,000 m and deep humid valleys in which the villages are situated. This ecosystem is very different from the dry Sahelian ecosystem of the African meningitis belt. Nonetheless, an outbreak of serogroup A meningococcal infections occurred in this zone, so we investigated the possible causes of this epidemic.

The introduction of the strain into this remote population, probably in February 2000, was almost certainly favored by intense commercial exchanges with surrounding populations during the coffee harvest period at the end of the dry season, when

roads are more navigable than during the rainy season. The epidemic strain then spread in the nonimmune population, which had no cohort immune barrier. All age groups had similar attack rates, in contrast to epidemics within the meningitis belt, which essentially affect children; the death rate in the absence of appropriate treatment was 100%. We showed that in 1999 to 2000 in Yaoundé, a large city situated in the tropical rainforest at about 600 km south of the meningitis belt, *N. meningitidis* was isolated in 13.4% of cases of bacterial meningitis, and most of the strains isolated belonged to serogroup A (3). Serogroup A and W135 meningococcal meningitis increased in Yaoundé between 1995 and 2000, possibly attributable to increases in human exchanges between the northern provinces (situated within the meningitis belt) and the central and southern provinces (6).

Other trigger factors frequently considered responsible for epidemics within the African meningitis belt are drought and the "Harmattan" wind because all major epidemics start at the driest period of the dry season and stop with the first rains. The Harmattan wind rarely reaches South Cameroon. Precipitation has been recorded over a number of years at Fontem Missionary Hospital. From 1995 to 1999, yearly rainfall averaged 2,300–2,500 mm, with only 0–50 mm from November to March. In the past 5 years, an average of no more than two consecutive months have been without rain, whereas almost four consecutive months without rain (December to the end of March) occurred just before the epidemic.

Thus, this outbreak appeared to result from several factors: 1) a virulent serogroup A strain belonging to ST-7 that had been responsible for recent epidemics in surrounding countries and was circulating in Cameroon; 2) the expansion of this strain, favored by the absence of an immune barrier in the population and by commercial exchanges; and 3) an exceptionally dry season. Outbreaks of meningococcal disease are not strictly bound to certain ecologic conditions occurring within the meningitis belt but may break out elsewhere. Since the epidemic reported here, another meningococcus A epidemic (~200 cases) has occurred at a similar equatorial latitude, near Bamenda (approximately 100 km north of Fontem), in 2001 (J. Kamgno, pers. comm.).

Health authorities should be aware of the possibility of such epidemics, be ready to alert medical practitioners and the public about them as they occur, and ensure that patients receive proper treatment and vaccines in these zones.

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West Nile Virus Meningitis in Patient with Common Variable Immunodeficiency

To the Editor: Infection by West Nile virus (WNV) was first recognized in the Western Hemisphere in 1999 in New York (1). Subsequently, this mosquito-borne flavivirus has spread westward and has emerged as an important cause of infectious meningoencephalitis in the United States (2). In September 2002, during a WNV epidemic in Michigan (2), a 38-year-old woman with common variable immunodeficiency (CVID) sought treatment at the University of Michigan Hospital with acute WNV-associated meningitis. Although persons with CVID are at increased risk for enteroviral meningoencephalitis, a greater susceptibility to arthropod-borne flavivirus infections has not been reported.

The patient had a history of recurrent sino-pulmonary infections and gastrointestinal giardiasis and salmonellosis; at 33 years of age, she was diagnosed with CVID that has been subsequently treated with intravenous immunoglobulin (IVIG) every 3 weeks. She was in her usual state of health until 5 days before admission, when she noted the abrupt onset of severe headache, followed by temperatures up to 39.4°C, progressive photophobia, nausea, vomiting, and a

transient papular rash on her trunk and extremities.

On arrival, the patient reported marked photophobia. Physical examination showed a temperature of 40.6°C, heart rate 80 beats per minute, and blood pressure 122/70 mmHg. She had cervical tenderness to palpation and active range of motion with minimal rigidity. Small, diffuse, nontender lymphadenopathy was noted in the cervical region. No focal or global deficits were found on neurologic exam. Results of the remainder of the physical examination was unremarkable. Initial laboratory values included a peripheral leukocyte count of 3.5 K/mm^3 (normal: 4.0–10.0 K/ mm^3; 42% neutrophils, 52% lymphocytes, and 7% monocytes), which was unchanged from the patient's baseline leukopenia. Her serum IgG level was 1,081 mg/dL (620–1,520 mg/dL).

Cerebrospinal fluid (CSF) sampling indicated the following: erythrocytes 3/mm^3, leukocytes 77/mm^3 (41% neutrophils, 51% lymphocytes, 7% histiocytes), glucose 50 mg/dL (50–70 mg/dL), and protein 75 mg/dL (15–45 mg/dL). Results of routine Gram stain, bacterial and fungal cultures, polymerase chain reaction testing for herpes simplex virus and *Cryptococcus neoformans* antigen were negative. Assay results of the patient's CSF for WNV by IgM-capture enzyme-linked immunosorbent assay, performed by the Michigan Department of Community Health, were positive.

The patient was initially treated with parenteral ampicillin, ceftazidime, and acyclovir, which were discontinued within 48 hours. Her symptoms improved with routine medical support, and she was discharged on hospital day 5. We were notified of the positive CSF IgM for WNV approximately 2 weeks after the patient was discharged, at which time her symptoms had completely resolved. At a follow-up visit 3 weeks after her hospitalization, the patient had no residual symptoms of meningitis.

Patients with agammaglobulinemia, either common variable or X-linked, are known to be susceptible to recurrent infections (3). Bacterial infections are the best described; however, chronic enteroviral meningoencephalitis is also associated with deficiencies in B-cell immunity (4–6). Although the role of immunoglobulins in host defense against WNV infection is not completely understood, evidence suggests that humoral immunity protects against WNV infection and severe disease (7–9).

WNV is a single-stranded RNA virus of the family *Flaviviridae*. Its genome is processed to eight proteins, including the envelope (E) glycoprotein, the matrix protein, the nucleocapsid protein, and five nonstructural proteins (7). E-glycoprotein antibodies develop during human WNV infection (8), and passive immunization of mice with E-glycoprotein antiserum protects against WNV infection and death (7,8). In addition, IVIG therapy was associated with recovery from WNV meningoencephalitis of an immunosuppressed 70-year-old woman (9). Although our patient had normal levels of serum IgG at the time of illness, viral meningitis may occur in agammaglobulinemic patients despite regular IVIG therapy (10).

This case demonstrates the need to consider WNV in patients with CVID. Our patient recovered promptly, without evidence of neurologic sequelae, despite her underlying immunodeficiency. More experience is needed to provide a better understanding of the relationship between CVID and WNV.

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Isolation of *Enterobacter sakazakii* from Midgut of *Stomoxys calcitrans*

To the Editor: *Enterobacter sakazakii,* a gram-negative rod-shaped bacterium, is an emerging foodborne pathogen that can cause meningitis, sepsis, or necrotizing enterocolitis in newborns, particularly affecting premature or other immunocompromised infants. Although an environmental reservoir and mode of transmission for *E. sakazakii* has not been clearly identified, a growing number of reports suggest that powdered milk–based infant formulas can be a vehicle for infection. We report the isolation of *E. sakazakii* from the guts of larvae of the stable fly, *Stomoxys calcitrans,* demonstrating an environmental reservoir for *E. sakazakii* and raising the possibility that environmental contamination by insects may be important in the spread of this opportunistic organism.

The first two cases of neonatal meningitis caused by *E. sakazakii* were reported in the United Kingdom in 1961; both infants died (1). Subsequently, cases of meningitis, septicemia, necrotizing enterocolitis, brain abscesses, cerebral infarctions and dermoid cysts of the posterior fossa caused by *E. sakazakii* have been reported worldwide.

Although most documented cases involve infants, reports also describe adult infections. Adults with *E. sakazakii* infections usually have serious underlying diseases or malignancies, but adult meningitis caused by *E. sakazakii* infection has never been reported. Concern is growing regarding antibiotic resistance in *E. sakazakii*. Recently, an *E. sakazakii* wound infection in an adult patient has been reported, which was resistant to multiple antibiotics and required prolonged treatment with broad-spectrum antibiotics (2).

Illness and death associated with *E. sakazakii* infection in neonates vary considerably, and mortality rates as high as 80% have been recorded (3). Implicated infection sources include incubators, the birth canal, a blender in a milk kitchen where powdered infant formula was reconstituted and hospital environments (*E. sakazakii* growth on an agar plate in a ward specifically under infection control against multidrug-resistant *Staphylococcus aureus* [MRSA]). The presence of *E. sakazakii* in powdered infant formula has been reported often. An investigation of Enterobacteriaceae found in powdered substitutes for breast milk from 35 countries indicated that 52.2% of the 141 samples tested contained members of this family. *E. agglomerans, E. cloacae, E. sakazakii,* and *Klebsiella pneumoniae* were the most frequently isolated organisms (4). More recently, *E. sakazakii* has been detected in ultra–heat-treated milk, spoiled tofu, lettuce, and traditional fermented bread (khamir), and in beer mugs rinsed mechanically or in open vats. Although more vehicles for transmission are being identified, the environmental reservoir has remained elusive. A study in 1990 did not isolate this organism from samples of surface water, mud, soil, rotting wood, bird dung, grain, rodents, domestic animals, cattle, or raw cow's milk (5). We report the isolation of *E. sakazakii* from the gut of larvae of the blood-sucking insect *Stomoxys calcitrans*.

S. calcitrans were reared in a laboratory culture (originally isolated from farms in North Wales, U.K.) as previously described (6). Larvae were removed from culture medium, washed in sterile isotonic saline solution, and surface sterilized by immersion in 100% ethanol. Larval guts were dissected aseptically and placed individually onto standard LB agar plates. Plates were incubated at 24°C overnight and were examined for the presence of bacteria the next day. Subcultures of each different colony type were streaked onto fresh LB plates until pure colonies were obtained.

A sample was taken from each pure colony and an attempt was made to identify the bacteria by using standard polymerase chain reaction (PCR) amplification of the genes encoding bacterial 16S rRNA (7). We identified *E. sakazakii*, *Providencia stuartii*, *Erwinia carotovora, Micrococcus luteus*, and *Serratia marcescens.* The *E. sakazakii* colony had the typical yellow pigmentation and both colony morphologic features reported for this bacterium (3).

The growing reports of *E. sakazakii* infections and its implied role as an emerging foodborne pathogen are of concern to clinicians, the food industry, and consumers. The high mortality rate and severity of this infection in infants, coupled with the lack of ecologic information about this organism, have fueled much debate. The voluntary product recall of a powdered infant formula by its manufacturer in 2001, after *E. sakazakii* was found in the finished product (8), exemplifies the need for stringent process control and the use of aseptic techniques during preparation, handling, storage, and use of reconstituted infant milk. The problem of foodborne transmission is further exacerbated by the high tolerance to heat of *E. sakazakii* (3). The overall risk for infection may depend on several factors, including numbers of bacteria present in the product, handling after preparation, and underlying patient characteristics.

We have identified the larval gut of the stable fly, *Stomoxys calcitrans,* as an environmental reservoir for *E. sakazakii*. Stable flies have a worldwide distribution; they are blood feeders that primarily feed on cattle, horses, dogs, pigs, and humans, but they will also take a blood meal from

reptiles and birds. *Stomoxys* spp. may be found wherever cattle, pigs, or horses are kept, and more specifically, *S. calcitrans* is common in cowsheds, making contamination of milk a possibility.

The geographic distribution of *S. calcitrans* correlates well with the incidence of *E. sakazakii* infections. Both *S. calcitrans* and *E. sakazakii* infections have been reported from Denmark, the United States, Israel, the United Kingdom, and Germany. Additionally, *Stomoxys* spp. have a worldwide distribution and are likely present in all countries reporting *E. sakazakii* infection.

A recent study has isolated *E. sakazakii* from the guts of a laboratory colony of Mexican fruit flies, *Anastrepha ludens* (9). Furthermore, a technical report from a pest exterminating company in the United States records the presence of *Enterobacter sakazakii* in the house fly (*Musca domestica*), although the exact location of the bacterium on or in the fly is unclear. Insects are thus a likely major environmental reservoir of *E. sakazakii*. This conclusion suggests that in addition to stringent control measures during manufacturing and use of foodstuffs, reducing arthropod presence in hospital and manufacturing environments could result in a substantial reduction in the transmission of *E. sakazakii*. New control methods will likely need to be developed because some evidence suggests that insect traps that electrocute could play a role in the spread of infectious disease agents such as bacteria and viruses (10).

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Acknowledgments

This study was supported by grants from BBSRC and The Wellcome Trust.

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Exotic Viral Diseases: A Global Guide

Stephen A. Berger,
Charles H. Calisher,
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BC Decker, Inc.,
Hamilton, Ontario, Canada, 2003
ISBN: 1-55009-205-7
Pages: 252 Price: $29.95

Exotic Viral Diseases: A Global Guide by Berger et al. is a small, softcover, 252-page handbook organized by disease or disease group. It addresses 55 viral pathogens, including a few broad groupings, such as "New World hantaviruses."

Because of increasing travel, which is eroding geographic barriers to disease transmission, and because of the emergence and reemergence of uncommon infectious diseases, frontline clinicians are increasingly more likely to encounter patients with exotic viral infections. The arrival of West Nile virus and monkeypox virus in North America demonstrates the potential for importation of unusual viral pathogens.

An 8-page overview of the assessment and evaluation of febrile viral syndromes precedes a series of four tables listing syndrome complexes, animal reservoirs, infectious vectors, and routes of infection. The tables are followed by sections describing each pathogen in alphabetical order. Each section includes common and generic designations for the virus, reservoirs, vectors, modes of transmission, incubation periods, "clinical hints," "typical therapy," and geographic distribution of the disease. Concise subsections provide general information, describe the clinical presentation and course of the illnesses, and recommend diagnostic procedures and laboratory biosafety levels. Sections end with a list of additional reading, ranging from 3 to 22 citations, including journal articles, book chapters, and electronic citations. In addition to the tables noted above, 10 charts are distributed throughout the sections, demonstrating disease incidence during recent decades. The book is indexed and has a 10-page appendix of drugs and vaccines and a 5-page appendix of diagnostic tests. It has no illustrations. An accompanying mini-CD ROM contains the same material as the printed edition.

Though the handbook's coverage of topics is superficial, its format makes this a useful quick reference, for example, as a reminder for clinicians assembling differential diagnosis lists for a febrile viral syndrome. The vector and syndrome tables are handy, and the disease descriptions contain sufficient information for preliminary consideration. However, this is not an exhaustive guide for clinical care and, as pointed out by the authors, does not obviate consultation of a more substantial reference or an expert in the management of a specific disease. Limited attention is given to isolation and infection-control recommendations. The CD ROM is a convenient feature, for example, for field use; however, more informative disks that accompany larger virology texts would probably have greater utility in such a setting.

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Conference Summary

Drug-resistant *Streptococcus pneumoniae* and Methicillin-resistant *Staphylococcus aureus* Surveillance[1]

The Centers for Disease Control and Prevention (CDC) convened a conference on March 12–13, 2003, in Atlanta, Georgia, to discuss improving state-based surveillance of drug-resistant *Streptococcus pneumoniae* (DRSP) and methicillin-resistant *Staphylococcus aureus* (MRSA). The Council of State and Territorial Epidemiologists, the Association of Public Health Laboratories, and CDC co-sponsored the conference; 120 participants from 38 states attended. The conference was organized by the Divisions of Healthcare Quality Promotion and Bacterial and Mycotic Diseases, National Center for Infectious Diseases, CDC. Goals of the meeting included 1) reviewing the rationale for surveillance of DRSP and MRSA, 2) presenting scientific studies highlighting valid and meaningful methods of performing state-level surveillance, 3) sharing state-level surveillance experiences, and 4) identifying unmet needs of the state health departments in performing such surveillance.

The primary theme of the conference general sessions was the public health impact of DRSP and MRSA and the need for accurate surveillance data to track and monitor resistance trends. *S. pneumoniae* is a major cause of respiratory infections in the United States. Since the early 1990s, the prevalence of resistance to single and multiple antibiotics has been increasing in pneumococci. Antimicrobial drug resistance in *S. pneumoniae* can vary among populations and is influenced by local prescribing practices and the prevalence of resistant clones. Conference presenters discussed the role of surveillance in raising awareness of the resistance problem and in monitoring the effectiveness of prevention and control programs. National- and state-level epidemiologists discussed the benefits of including state-level surveillance data with appropriate antibiotic use programs designed to address the antibiotic prescribing practices of clinicians. The potential for local surveillance to provide information on the impact of a new pneumococcal vaccine for children was also examined; the vaccine has been shown to reduce infections caused by resistance strains

Since the early 1990s, *S. aureus* infections resistant to oxacillin (MRSA) have increased steadily. Several scientists reported two recent changes in the epidemiology of MRSA: its emergence in persons without established risk factors and the emergence of vancomycin-resistant *S. aureus* (VRSA). These new developments underscore the need for scientifically valid, yet financially feasible, state-based surveillance that will aid in understanding the changing epidemiology of MRSA disease. Such understanding will allow effective implementation of MRSA prevention and control programs. Prevention programs will differ on the basis of which populations are most affected (long-term care, community, and hospice). Current programs focus on reducing cross-transmission through improved hand hygiene and wound care. (More information is available from: URL: http://www.cdc.gov/ncidod/hip/Aresist/aresist.htm).

Conference participants generally agreed that a lack of resources for surveillance has challenged state public health agencies committed to monitoring emerging antimicrobial drug resistance. A conclusion drawn from conference sessions was that statically sound methods of data collection that capture valid, meaningful, and useful data and meet the financial restrictions of state budgets are indicated.

Active, population-based surveillance for collecting relevant isolates is considered the standard criterion. Unfortunately, this type of surveillance is labor-intensive and costly, making it an impractical choice for many states. The challenges of isolate collection, packaging and transport, data collection, and analysis may place an unacceptable workload on laboratory and epidemiology personnel.

Epidemiologists from several state health departments that have elected to implement enhanced antimicrobial drug-resistance surveillance programs presented alternative surveillance methods currently implemented in their states. Several surveillance models and knowledge gained by state-based epidemiologists provided key insights into the challenges and benefits of implementing enhanced surveillance programs.

Two methods frequently used by states are sentinel (i.e., survey of subset of laboratories) and antibiogram (i.e., cumulative susceptibility data) surveillance. Common difficulties were identified with implementing sentinel systems. Those difficulties included logistical obstacles with isolate or data processing and communication breakdowns between laboratory, epidemiology, and hospital infec-

[1]Presenters included the following: Richard E. Besser, Centers for Disease Control and Prevention (CDC); Clare Kioski, Arizona Department of Health Services; Cynthia G. Whitney, CDC; Scott K. Fridkin, CDC; Kathleen LeDell, Minnesota Department of Health; Elizabeth Bancroft, Los Angeles County Department of Health Services; Scott Seys, Wyoming State Health Department; Felicia Medella, Nebraska Health and Human Services; Chris A. Van Beneden, CDC; and Norman Crouch, Public Health Laboratory, Minneapolis, Minnesota.

tion control personnel. Care must be taken in selecting the numbers and types of laboratories to participate in the sentinel network States collecting antibiograms from hospitals and state laboratories also face challenges, including incompatible formatting of drug-testing panels, the inconsistent inclusion of duplicate or repeat isolates, and inconsistent reporting of denominator data. Solutions to these problems commonly involve improving communication between clinical microbiology laboratories and state health departments, including laboratory input in decision making and providing feedback of data from the system to participants. Guidance for aggregating cumulative susceptibility data (i.e., antibiograms) has been published and can serve as a guide for states and clinical microbiology laboratories in conducting surveillance. Also, having designated staff was essential for successfully implementing most programs. Presenters agreed that the benefits of collecting local data from these systems are substantial and will assist prevention programs.

Another aspect of surveillance focuses on detecting rare events. Such reports may include new changes in susceptibility, new mechanisms of resistance, susceptibility of unusual pathogens, and unexpected sources of resistant organisms. Establishing good communication among personnel in health departments and clinical laboratories is important for improving reporting of such events.

Allocating resources for improved surveillance is considered a practical and responsive step for states interested in tracking local resistant trends. Local data are important for raising public awareness, establishing resources and prevention activities, developing and informing treatment guidelines, monitoring trends, and motivating behavior change among clinicians.

This meeting and ongoing efforts to study and validate surveillance methods will assist local health authorities in making decisions programs to monitor antimicrobial drug resistance. The 2-day conference provided an opportunity to initiate an exchange of current practices and knowledge gained among states and territories. This process marks the first phase in building networks that may potentially enhance training resources, provide guidance for program development, and identify further technical assistance needed from CDC.

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Jacques-Louis David (1748–1825). Coronation of Empress Josephine by Napoleon I at Notre Dame de Paris, 2 December 1804 (1806–1807)
Detail of Napoleon and Josephine. Oil on canvas, 6.1 m x 9.31 m. Photo: Peter Willi
Réunion des Musées Nationaux/Art Resource, NY. Chateaux de Versailles et de Trianon, Versailles, France

"I was always hiding behind the instructor's chair, drawing for the duration of the class," admitted Jacques-Louis David, acknowledging his early artistic bent (1). The orphaned son of a wealthy Paris family, David went on to study art, first under distant relative François Boucher, then under gifted teacher and rococo painter Joseph-Marie Vien. Later, in Italy for 5 years, David became engrossed in archaeology, classical architecture, and mythology, which, along with the paintings of his compatriot Nicolas Poussin, provided inspiration for his work as leading neoclassical painter (2).

David's era was the Age of Enlightenment, whose standard-bearers (David Hume, Voltaire, Jean-Jacques Rousseau, Heinrich Heine, and others) revolutionized economics, politics, and religion, steering them away from authoritarian tradition, toward reason and the common good. The arts, abandoning the baroque, relinquished the ornate, aristocratic, and frivolous excesses of rococo. They turned toward nature and heroic morality, and by extension, toward the ancient "apostles of reason," the classics, and their "noble simplicity and calm grandeur." Italy's artistic leadership declined, leaving the role of guardian of Western art to France and the students of Vien (3).

The great political upheavals of the mid-18th century, the American Revolution and the French Revolution, followed the sweeping changes in the world of ideas and ushered in the modern era. David enthusiastically took part in the French Revolution and interpreted the issues of his day in masterpieces drawn from ancient moral dilemmas (The Death of Socrates) and contemporary events (The Death of Marat).

Napoleon Bonaparte rose to power after a coup d'état in 1799. A plebiscite in 1802 confirmed his lifetime rule as Consul of France, in preparation for his becoming Emperor of the French Republic. In 1804, in an elaborate ceremony reminiscent of the coronation of Holy Roman Emperor Charlemagne and in the presence of Pope Pius VII, Napoleon grasped his sword to his heart and put the crown on his own head (4). David, then official painter to the emperor, was tasked with commemorating the coronation festivities at the cathedral of Notre Dame. David's rendition of the event, on this month's cover of Emerging Infectious Diseases, does not dwell on Napoleon's imperial indiscretion. Rather, it portrays the crowning, by the emperor, of his wife Josephine. Josephine's coronation itself is a small part of the massive composition, an enormous group portrait of more than 100 figures (5).

Art has been a powerful instrument of revolutions, and David used it often to portray Napoleon as legendary opponent of absolutism, embodying the quest for truth and liberty. Music has similarly served popular uprisings. Beethoven's Third Symphony, "Eroica," for a brief time referred to as the Bonaparte Symphony, was inspired by Napoleon's heroic promise. This epic symphony, which dramatically captures the spirit of humanity, parallels the complexity of revolutionary passions (6).

In the eyes of the world, as in the eyes of those attending the lavish coronation in David's painting, Napoleon's fall came the moment he assumed imperial status. The laurel leaf crown of Roman emperors was no simple corona. To the champions of equality it symbolized tyranny. David's political entanglement with the Napoleonic era ended in imprisonment and exile. The dedication to Napoleon in Beethoven's score of the "Eroica" was retracted. And Josephine was banished to make room for Napoleon's true mistress, power.

The crown and its elusive promise have downed many a revolutionary hero, the corona of power often becoming halo of disaster. The same is true at times in nature. Some biologic agents are reminiscent of the sun, whose corona is only visible during a full eclipse. The coronaviruses, named for their crownlike appearance in which a loosely wound center is neatly surrounded by club-shaped peplomers, are a case in point. Known animal pathogens for many years, the more than 15 species of coronaviruses infected a variety of mammals and birds yet remained largely obscure, until antigenic variation or some other, unknown, cause brought them into the spotlight. An animal pathogen causing zoonotic infection in humans or a recombinant of human coronavirus and animal virus, severe acute respiratory syndrome virus has brought on a halo of disaster, circling the globe with illness and death.

Polyxeni Potter

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Upcoming Infectious Disease Conferences

October 7–8, 2003
Intensive Update Course
in Clinical Tropical Medicine
and Travelers' Health
American Society of Tropical
Medicine and Hygiene
San Diego, CA
Contact: ASTMH
Phone: 847-480-9592
email: astmh@astmh.org
Web site: http://www.astmh.org

October 9–12, 2003
Infectious Diseases Society of
America (IDSA)
San Diego, CA
Contact: 703-299-0200
email: info@idsociety.org
Web site: www.idsociety.org

October 26–29, 2003
Symposium on Bluetongue
Office International des Epizooties
Taormina, Italy
Contact: 33 (0)1 44 15 18 88
email: oie@oie.int
Web site: www.oie.int

November 9–13, 2003
6th OIE Seminar on Biotechnology
and 11th International Symposium of
the World Association of Veterinary
Laboratory Diagnosticians
Office International des Epizooties
Bangkok, Thailand
Contact: 33 (0)1 44 15 18 88
email: oie@oie.int
Web site: www.oie.int

November 15–19, 2003
131st Annual Meeting, American
Public Health Association
San Francisco, CA
Contact: APHA Convention Services
(202) 78-05600
Web site: http://www.apha.org

EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.9, No.11, November, 2003

Upcoming Issue

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

Look in the November issue for the following topics:

Risks and Benefits of Pre-and Post-Exposure Smallpox Vaccination

Cardiac Deaths after 6 Million Smallpox Vaccinations, New York City, 1947

Toxoplasma gondii Infection in the United States, 1999-2000

Toxoplasma gondii and Schizophrenia

Coronavirus-positive Nasopharyngeal Aspirate as Predictor for Severe Acute Respiratory Syndrome Mortality

West Nile Virus Infection in Nonhuman Primate Breeding Colony, Concurrent with Human Epidemic, Southern Louisiana

Rapid Antigen Capture Assay To Detect West Nile Virus in Dead Corvids

Severe Acute Respiratory Syndrome-associated Coronavirus Infection

Fluoroquinolone-resistant *Salmonella enterica* Typhimurium in Humans

Cowpox with Severe Generalized Eruption, Finland

Flow Cytometry and T-Cell Response Monitoring after Smallpox Vaccination

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. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases website (www.cdc.gov/eid).

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.