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Georgia O'Keeffe (1887-1986). Cow's Skull with Calico Roses (1932).
Oil on canvas (91 cm x 61 cm). The Art Institute of Chicago

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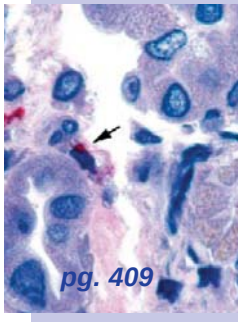

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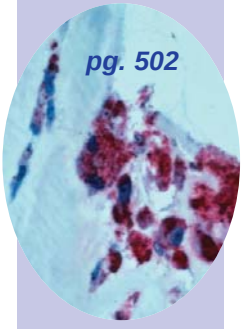

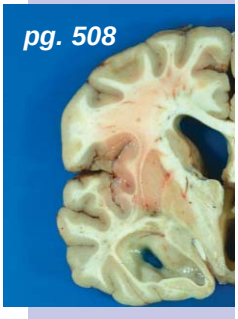

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Clinical Trials and Novel Pathogens: Lessons Learned from SARS

Matthew P. Muller,*† Allison McGeer,*† Sharon E. Straus,†‡ Laura Hawryluck,†‡ and Wayne L. Gold†‡

During the recent global outbreak of severe acute respiratory syndrome (SARS), thousands of patients received treatments such as ribavirin and corticosteroids. Despite this, no controlled clinical trials assessing the efficacy of these agents were conducted. If a second global SARS outbreak occurred, clinicians would not have controlled data on which to base therapeutic decisions. We discuss the unique methodologic and logistical challenges faced by researchers who attempt to conduct controlled trials of therapeutic agents during an outbreak of a novel or unknown infectious pathogen. We draw upon our own experience in attempting to conduct a randomized controlled trial of ribavirin therapy for SARS and discuss the lessons learned. Strategies to facilitate future clinical trials during outbreaks of unknown or novel pathogens are also presented.

The recognition of SARS as a transmissible disease prompted international efforts to identify its cause and control its spread. The success of these efforts has been dramatic, with the identification of the SARS-associated coronavirus (SARS-CoV) and the control of SARS outbreaks in all affected countries (1–4). An evidence-based approach to managing SARS is still lacking, however, as no controlled clinical data are available to justify any of the treatments used. If SARS reemerges, clinicians will have little evidence on which to base treatment decisions. Could clinical trials have been conducted during the global outbreak? If so, what steps need to be taken to ensure that such trials are implemented appropriately the next time a similar event occurs?

We highlight the challenges faced by researchers attempting to conduct clinical trials of therapeutic agents during an outbreak caused by an unknown or novel pathogen. We focus the discussion on the design and implementation of randomized controlled trials of candidate therapeutic agents, as such trials are the standard on which therapeutic decision-making should be based. Examples from our own experience attempting to launch a randomized trial of ribavirin therapy for SARS will illustrate these challenges.

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Formulating the Research Question

The first step in conducting a clinical trial is to develop a simple, testable hypothesis. The challenge facing researchers at the beginning of an outbreak caused by an unknown or novel pathogen is selecting a hypothesis in the face of scarce but rapidly evolving information. Once a hypothesis is selected and a trial started, new information arising during the outbreak may undermine the study hypothesis before the trial is completed.

In the initial phases of the SARS outbreak, empiric therapy was used to provide coverage against a broad differential of bacterial and viral pathogens. Ribavirin was included for coverage of a presumptive viral illness, given its in vitro activity against a variety of RNA and DNA viruses (5). As data on pathogenesis accrued, a hypothesis suggesting that lung injury may be immune mediated led to widespread use of corticosteroids in combination with ribavirin (6,7). Finally, isolation of SARS-CoV allowed for in vitro susceptibility studies that, in combination with increasing reports of toxicity attributed to ribavirin, resulted in discontinuation of ribavirin as a treatment for SARS in Toronto, although not in all affected areas (8,9).

Presently, a randomized trial of corticosteroids appears to be a rational direction for future treatment trials, given their widespread use in the treatment of SARS, despite minimal supportive evidence, and their potential risks. Early in the outbreak, however, we focused our attention on designing a trial to evaluate the efficacy of ribavirin, which was being used in Canada and Hong Kong and had been associated with clinical successes in uncontrolled reports (10,11). We believed that conducting such a trial was important, given the widespread use of ribavirin and its potential risks and benefits. Subsequently, reports of adverse events associated with its use, demonstrated lack of in vitro activity, and reports of clinical progression on therapy convinced Canadian clinicians to discontinue its use without further study (8,9,12). At this point, we do not feel that a randomized trial of ribavirin therapy is

warranted. Some may argue that such a trial would have been valuable, given the continued use of ribavirin for the treatment of SARS in Hong Kong and China (13).

The hypothesis selected for evaluation during an outbreak must address an important clinical question that cannot be rapidly answered by other means. Although there may be a temptation to launch a trial rapidly after the onset of an outbreak, in most cases the etiologic agent should be identified with reasonable certainty before conducting trials of specific agents. Agents selected for study should have plausible mechanisms of action, activity established by in vitro studies, known toxicity profiles, and preliminary clinical evidence supporting their efficacy. Evidence should not consist solely of anecdotal case reports but should include some data derived from comparisons of treated and untreated patients. If other means of rejecting the hypothesis exist (i.e., in vitro studies, animal studies), but have not yet been conducted, a trial of the agent should be deferred and its resources allocated to trials testing agents that cannot be evaluated by other means. For example, the hypothesis that steroids can improve outcome in SARS by preventing or abrogating immune-mediated lung injury will be difficult to disprove by any means short of a randomized controlled trial.

If a randomized trial is not considered justified, feasible, or ethical, therapeutic agents of unknown efficacy should not be used without attempts to systematically document illness severity, clinical course, treatment, adverse events, and confounding factors in a standardized manner to facilitate analysis. At a minimum, comparisons of cohorts of patients treated differently may yield information that can inform subsequent trials.

Identifying the Study Population

In all clinical trials, the patient population to be included must be precisely defined. In an outbreak involving an unknown or novel pathogen, specific microbiologic tests to diagnose infection will not be available. Case definitions used by public health authorities to identify patients and to limit transmission through isolation and quarantine are deliberately broad to ensure that few contagious persons are missed. As a result, the specificity of diagnosis may be poor. Conducting a trial without a specific diagnostic test may result in the inclusion of patients without the condition of interest. These patients will receive potentially toxic therapies with no possible benefit. Furthermore, inclusion of patients without the disease of interest will bias the study towards the null hypothesis.

The initial case definitions for SARS are examples of this problem. Based on the World Health Organization's initial case definition, persons presenting with fever, respiratory symptoms, and an epidemiologically defined exposure are considered to have SARS (14). This definition is non-specific, as all patients with febrile respiratory illnesses, regardless of etiology, will be included if they have any exposure history. This was particularly problematic in returned travelers with respiratory illness. Because direct SARS exposure was uncommon among travelers, even to high-risk areas, inclusion of travel-related cases in our trial would have resulted in a low positive predictive value for the case definition.

Although no simple solution to this problem exists, the specificity of inclusion criteria may be improved by ensuring that appropriate microbiologic investigations are performed to exclude patients with alternative diagnoses and by narrowing the epidemiologic case definition. For example, in the case of SARS, limiting study inclusion to those with pulmonary infiltrates on chest radiograph and requiring that epidemiologic links be based on direct contact with another case of SARS (which would exclude the majority of travelers to SARS-affected regions) would improve specificity. However, one must be careful when specificity is increased by selecting patients with more advanced presentations of disease because these patients may be less likely to respond to therapy compared with patients identified earlier in their disease.

Finally, post-hoc analysis of trial results can be performed once specific microbiologic and serologic tests become available. This analysis will allow further assessment of therapeutic efficacy. However, every effort should be made during the trial to exclude patients with a low probability of having the disease of interest to minimize the likelihood of treating uninfected patients with potentially toxic agents. This will minimize the number of patients treated inappropriately and maximize the likelihood of identifying differences in outcomes between the treatment and control groups.

Defining the Intervention

All clinical trials require that the intervention used be defined explicitly. Dose, frequency, duration, and route of administration must be predetermined and are usually based on data from in vitro, animal, human safety, and dose-finding studies. In an outbreak involving an unknown or novel pathogen, these data will be unavailable. Choosing an intervention will be based on analogy to the treatment of other infectious diseases and available safety data. Negative trial results will be open to the criticism that a different dose or duration of therapy might have been effective.

In Toronto, the initial dose of ribavirin used was based on limited clinical experience with the use of high-dose ribavirin in the treatment of hemorrhagic fever syndromes (15). A similar dose was selected for our trial. Clinicians in Toronto who treated patients with SARS observed a high incidence of adverse effects associated with this regimen

(9,12). As a result, regimens using a lower dose emerged, and ribavirin therapy was ultimately abandoned. Had our initial trial been launched, clinicians would have been unwilling to enroll patients, due to the high doses of ribavirin proposed and the emerging reports of drug toxicity.

Ideally, interventions selected for a randomized trial should be based on preliminary data. Outbreaks of unknown and novel pathogens represent unique situations in which such data will not be available. The initial use of supportive care alone is a defendable position, particularly when minimal evidence exists to support the use of any therapeutic agent, and considerable evidence exists to support potential harm. This approach was adopted in the United States, and no fatal cases were reported, although only eight U.S. cases have been serologically confirmed (4). In parts of the world where outbreaks were larger, considerable pressure was placed on clinicians to offer specific therapies directed against SARS-CoV, even if such therapies were unproven and potentially dangerous. When unproven and potentially harmful therapeutic agents are being used in clinical practice, a simple, rapidly conducted randomized trial may be beneficial. Protocols will need to allow for the evolution of clinical practice during the outbreak. Although changes in dose or duration may make interpretation of results more difficult, rigid adherence to initial protocols may make trials unacceptable to clinicians and potentially dangerous to patients. Such trials require ongoing communication with trial participants and timely disclosure of emerging information about efficacy and safety.

Defining Study Outcomes

All clinical research requires a clearly defined and clinically relevant outcome. In an outbreak caused by an unknown or novel pathogen, the ultimate size of the outbreak will dictate enrollment and cannot be predicted in advance. Concerns that the trial will be underpowered are likely to arise. To enhance feasibility, selecting more frequently occurring, but less clinically relevant, outcomes may be tempting.

Estimates of case-fatality rates for the Toronto SARS outbreaks varied from 30%, to 3%, to 6.5%, and then to 17% as the outbreak progressed (9,16,17). Our initial sample size calculations using mortality as the outcome were based on a case-fatality estimate of 3% in the treatment (ribavirin) group. Using an alpha of 0.05 and a power of 80%, the sample size required to detect a 25% reduction in case-fatality rate from 4% in the placebo group to 3% in the ribavirin group would be 191 patients per group**.** Even if every SARS patient in Canada had been successfully enrolled, the study would not have recruited sufficient patients. Our solution was to use a composite outcome of death, mechanical ventilation, or severe hypoxemia to reduce the required sample size.

Due to the unknown size of an outbreak, the use of composite endpoints is a reasonable strategy to maximize the number of observed outcomes and minimize the required sample size, provided every component of the composite endpoint is clinically relevant and can be measured in a standardized manner.

Challenges to Recruitment

The feasibility of conducting a controlled trial during an outbreak is dependent on the ability to recruit sufficient numbers of patients into the study before the outbreak ends. In the first Toronto outbreak, SARS developed in 249 patients between the time the outbreak was recognized on March 13, 2003, and the end of the outbreak on April 25 (Figure). The decision to conduct a trial was made on March 23, 10 days after the recognition of the first cases of SARS. A protocol was rapidly developed and approved by the first local research ethics board on March 28 and by Health Canada on March 31. Review of the epidemic curve shows that the 18-day delay from the time the outbreak was recognized on March 13 to the receipt of expedited ethics approval on March 31 resulted in loss of 149 (60%) of 249 potential patients who could otherwise have been assessed for enrollment (Table). Cases that occurred during the second Toronto outbreak were not considered, as the use of ribavirin had been rejected by clinicians. Even if the second outbreak was considered, few additional case-patients could have been recruited because increased knowledge about control strategies resulted in reduced transmission and a smaller outbreak.

The ability to launch a trial quickly in the face of an outbreak is dependent on the speed of protocol development and the time required for obtaining ethics approval and study funding. Strategies that could be developed before the next outbreak of an unknown or novel pathogen to facilitate the rapid initiation of trials include the estab-

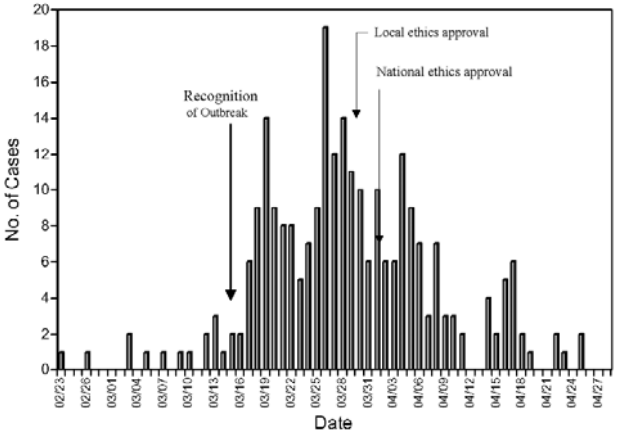

Figure. Epidemic curve of the first Toronto SARS outbreak. Data provided courtesy of the Ministry of Health and Long Term Care, Ontario, Canada.

Table. Eligible cases lost to enrollment in a hypothetical trial of ribavirin during the first outbreak of SARS in Toronto.

Date from outbreak recognition to date of enrollment of first patient (days)	Number of cases lost to enrollment (%)
March 13–16 (3)	6/249 (2)
March 13–20 (7)	37/249 (15)
March 13–23 (10)	62/249 (25)
March 13–28 (15)[a]	114/249 (46)
March 13–31 (18)[b]	149/249 (60)

[a]Approval from first local ethics review board
[b]Approval from Health Canada

lishment of a collaborative multicenter research network, the creation of a contingency fund for urgent therapeutic trials, and the development of new processes for emergency expedited ethics review.

Value of a Collaborative Research Network

A national or international collaborative research network consisting of infectious disease clinicians, microbiologists, epidemiologists, and clinical trial methodologists should be established. The network would facilitate the rapid initiation of trials during an outbreak through the advanced preparation of study protocols and by providing urgent assistance in the design and implementation of trials after recognition of an outbreak. A major role of the network would be to establish a communication system to connect front-line clinicians and network collaborators at multiple sites to facilitate an exchange of clinical information. This would allow accumulated clinical experience to be shared between sites and would provide a forum for the discussion of patient management issues. Furthermore, it could be used to identify and select hypotheses for evaluation, determine the feasibility of studies, and recruit clinicians to participate in trial design and implementation. The network would shoulder the burden of protocol development and implementation, including liaising directly with local or national ethics boards, thereby reducing workload on the clinicians who will be fully occupied with clinical responsibilities.

Contingency Fund for Urgent Therapeutic Trials

Randomized trials are resource intensive and cannot be initiated without financial support. Outbreaks are unpredictable events that place enormous strains on healthcare systems. To launch a trial promptly during an outbreak, without diverting local funding away from efforts that are crucial for outbreak control, a centralized contingency fund for urgent clinical therapeutic trials should be established nationally or internationally. Ideally, funding would support an external clinical trials team capable of operationalizing the trial, as local personnel may be overwhelmed by clinical responsibilities related to the outbreak.

Research Ethics Boards and Ethics Approval

The need to obtain approval from research ethics boards is another factor that may delay initiation of a trial. This requirement is further complicated by the mobile nature of outbreaks. Individual institutions may be affected by an outbreak for limited periods of time; the outbreak may spread to new geographic areas and involve new institutions. Investigators may need to repeatedly submit their protocol to new ethics boards, resulting in further delays and missed opportunities.

Our trial of ribavirin in SARS treatment was approved at several university-affiliated teaching hospitals involved in caring for patients during the first SARS outbreak in Toronto. By the time the trial was approved and ready to start, the outbreak appeared to be over. When a second outbreak occurred, it was centered at a hospital where ethics approval had not been obtained.

Several potential solutions to this problem exist. The most fundamental would be the creation of a national or regional emergency ethics review board with the authority to override the need for local institutional approval and the capability of rapidly assessing protocols when outbreaks occur. In certain circumstances, ethics approval could be obtained prior to an outbreak. For example, a protocol for a trial of corticosteroids in SARS could be designed and approved now and implemented if another outbreak occurs. Additionally, a "rolling" type of ethics approval could be developed, whereby portions of a trial could be approved while other elements are modified in real time as the trial is launched, with ongoing supervision and periodic reapproval by the ethics board. This would allow flexibility in refining dosing regimens and prevent rigid protocols from being repeatedly abandoned as new information emerges.

Risks to Healthcare Workers and Researchers

Transmissibility of pathogens is a feature unique to outbreaks of infectious diseases and has direct implications for conducting clinical trials. In an outbreak, both caregivers and researchers involved in assessing and monitoring patients may be placing themselves at risk. This situation is particularly true in the early stages of an outbreak involving a novel pathogen such as SARS because the most effective means of preventing transmission are not yet known. For example, SARS studies conducted in Toronto required clinicians to obtain specimens such as nasopharyngeal swabs in addition to those collected for clinical purposes. Such actions increase the potential for both exposure to and acquisition of SARS.

In addition to personal risk, researchers may become vectors of disease, placing patients, colleagues, family members, and institutions at risk. Immediately after ethics approval was obtained for our trial, one of the trial's primary investigators was hospitalized with SARS, and

several other researchers were quarantined. Although these exposures did not result from participation in the clinical trial, they highlight the difficulty of conducting clinical trials during an outbreak, especially when researchers are also involved in outbreak management and may be moving between institutions. Evidence that healthcare workers working part-time at several Toronto area hospitals transmitted SARS from one institution to another led to the public health requirement that healthcare workers be limited to a single institution. In particular, healthcare workers at hospitals in which transmission had occurred were not permitted to enter other hospitals. Such policies were important in limiting the spread of the outbreak but provide additional challenges to trial implementation.

In the future, investigators and research ethics boards will need to expand their horizons to consider the risks and benefits of trials as they apply to participating clinicians, researchers, and the public, as well as to the individual patient. In some cases, increasing the potential for exposure of either clinicians or researchers above what is clinically mandated may be considered unethical, particularly when novel pathogens of unknown mode of transmission are involved. Local ethics boards may not have the experience or expertise to deal with issues of public safety; this provides another argument for the creation of a specialized, national ethics board to deal with clinical trials during outbreaks or other public health emergencies.

Various approaches could be utilized to manage these risks. A cautious approach to preventing transmission and providing adequate training for participating clinicians and researchers should be required. Integrating clinical care and trial monitoring can reduce the number of patient visits. Sharing specimens between clinical and research laboratories may eliminate the need for additional specimen collections, and symptom assessment by telephone interview would minimize the need for patient contact. The study should be coordinated centrally and communication between study sites should be by teleconference and email, until risks are defined and minimized. Travel between centers would be limited by providing data collection forms designed to be directly scanned and transmitted via the Internet. This would reduce transmission risk and also allow the participation of multiple international sites in a single trial. Similar approaches have been effective in other areas of clinical research and can also increase study enrollment (18).

Conclusions

Clinicians caring for patients with infectious disease syndromes caused by unknown or novel pathogens are under intense pressure to offer potentially efficacious therapies. In future outbreaks, as in the SARS outbreak, therapeutic agents of unknown benefit will likely be used again. These agents should be used in the setting of randomized trials, to determine their efficacy and prevent therapies of unknown efficacy (e.g., corticosteroids for SARS) from becoming "standard of care" in the absence of good evidence.

The unpredictable nature of outbreaks poses many challenges to the successful design and implementation of such trials. Creation of national or international collaborative groups, with a mandate to implement clinical trials of therapeutic agents in outbreak settings, and supported by appropriate funding, may be the best strategy for achieving this goal. The collaboration of ethics review boards in establishing a process which facilitates trials while ensuring the safety of participating patients, researchers, and communities, is critical.

After the SARS outbreak in 2003, thousands of patients were treated with agents of unproven efficacy and definite toxicity; data on these agents' efficacy were not gathered. To prevent this situation from repeating itself, we must be prepared to conduct prospective, randomized controlled trials in the event of future outbreaks of novel pathogens.

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Dr. Muller trained in internal medicine and infectious diseases at the University of Toronto. Currently, Dr. Muller is completing a graduate degree in clinical epidemiology and is enrolled in the Clinician-Scientist Training Program, both at the University of Toronto. His current research interests include the epidemiology and treatment of severe acute respiratory syndrome and the epidemiology of antibiotic resistance.

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SARS Transmission and Hospital Containment

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An outbreak of severe acute respiratory syndrome (SARS) was detected in Singapore at the beginning of March 2003. The outbreak, initiated by a traveler to Hong Kong in late February 2003, led to sequential spread of SARS to three major acute–care hospitals in Singapore. Critical factors in containing this outbreak were early detection and complete assessment of movements and follow-up of patients, healthcare workers, and visitors who were contacts. Visitor records were important in helping identify exposed persons who could carry the infection into the community. In the three hospital outbreaks, three different containment strategies were used to contain spread of infection: closing an entire hospital, removing all potentially infected persons to a dedicated SARS hospital, and managing exposed persons in place. On the basis of this experience, if a nosocomial outbreak is detected late, a hospital may need to be closed in order to contain spread of the disease. Outbreaks detected early can be managed by either removing all exposed persons to a designated location or isolating and managing them in place.

Severe acute respiratory syndrome (SARS) has been characterized by efficient transmission in healthcare facilities, highlighting the vulnerability of our modern healthcare system to nosocomial infection (1,2). Frequent unprotected or inadequately protected patient-to-healthcare worker interactions (3) and grouping large numbers of ill persons can greatly amplify intrahospital transmission. If uncontrolled, SARS outbreaks in hospitals may rapidly degrade hospital services and can increase the risk for infection spread into the general community. Hence, rapid and effective containment of hospital SARS outbreaks is important.

In Singapore, an outbreak of SARS was started when a traveler (patient A) visited Hong Kong during February 20 to 25, 2003 (4). Patient A returned to Singapore and was admitted to an acute care hospital, Tan Tock Seng Hospital (TTSH), on March 1. Singapore was removed from the World Health Organization's list of areas with local SARS transmission on May 31. At that time, 206 probable SARS cases had been diagnosed, of which 40.8% were in healthcare workers; 39.8% were in family, friends, or visitors to hospitals; and 12.2% were in inpatients.

The outbreak in TTSH spread to two other tertiary hospitals (Singapore General Hospital [SGH], and National University Hospital [NUH]) despite initial containment efforts (Figure 1). The initial failure of the containment strategy was compounded by the presence, early in the outbreak, of three superspreading events, i.e., SARS patients directly associated with 10 or more secondary infections each. The Table summarizes the profile of the outbreak in the three hospitals. To contain the outbreak in Singapore, we used three separate hospital strategies: 1) closing the hospital, 2) removing an exposed group to a designated hospital, and 3) managing an exposed cohort in place. We review the three hospital containment strategies and the effectiveness of these strategies.

Sequential SARS Outbreaks

TTSH Cluster

The index case-patient, patient A, was admitted to TTSH, a 1,400-bed hospital, on March 1, 2003 for atypical pneumonia. She was treated in a six-bed ward (ward 5A) until she was isolated on March 6.

During the 6 days patient A was in ward 5A, 24 of her primary contacts were infected, and subsequently probable SARS developed in all. These included eight nurses, one health attendant, five patients in the same ward, and 10 visitors.

A second superspreading event occurred when one of the nurses infected by patient A, patient AA, was admitted

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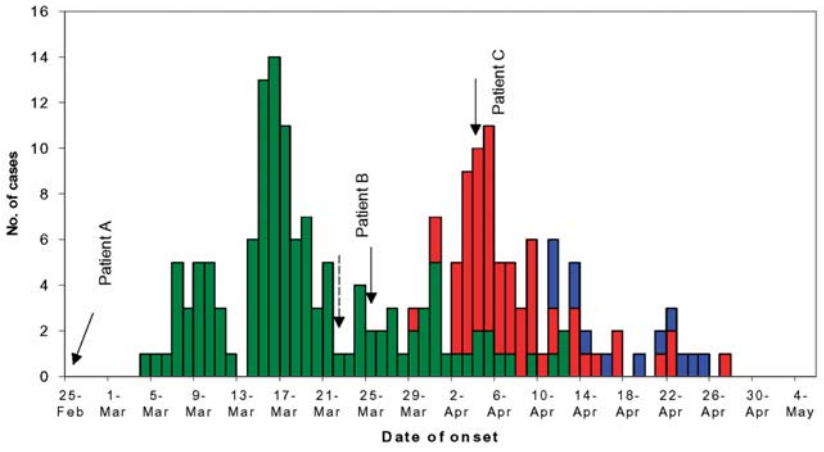

Figure 1. Severe acute respiratory syndrome case-patients infected at three major hospitals, Singapore, February–April 2003. The chart depicts the overall epidemic in each hospital, includes case-patients infected outside the hospital but whose disease origin was linked back to one of the three hospital outbreaks. In Tan Tock Seng Hospital (green), the last case of intrahospital transmission was on April 12. In Singapore General Hospital (red), the last case of intrahospital transmission was on April 15. In National University Hospital (blue), the last case of intrahospital transmission was on April 25. Arrows indicate dates of onsets of the three index cases for each hospital outbreak. Dotted arrow indicates date when full infection control measures (Figure 2) were implemented in Tan Tock Seng Hospital.

to an open ward (8A) on March 10. Patient AA was isolated on March 13, by which time 25 persons (12 healthcare workers, four patients in the same ward, eight visitors, and one household contact) had been infected. One of these patients (patient AAA) had multiple medical problems, including diabetes, gram-negative bacteremia, and ischemic heart disease that required her admission to the coronary care unit (CCU) from March 12 to 19. Patient AAA was not isolated for 8 days, resulting in a third superspreading event. Because of her multiple medical problems, SARS infection was not suspected, and healthcare workers caring for her did not use N95 masks. Twenty-seven people at the CCU were infected, including five doctors, 13 nurses, one ultrasonographer, one attendant, two cardiac technicians, and five visitors.

In addition to the spread of SARS within TTSH, infection also spread outside by infected visitors and discharged patients to household contacts and healthcare workers in another hospital, Changi General Hospital. The failure to detect SARS in a discharged patient (patient B) who was a contact of patient A in TTSH led to a second major outbreak in SGH, where this patient was subsequently readmitted (Figure 2).

Public Health Response

On March 15, probable SARS was diagnosed in a total of 13 persons, all of whom were admitted to TTSH. Six of these case-patients were family members and friends of patient A, six were healthcare workers who had attended to patient A in ward 5A, while one was a healthcare worker

Table. Key features of the outbreak in Tan Tock Seng Hospital, Singapore General Hospital, and National University Hospital

Features	Tan Tock Seng Hospital (N = 109)	Singapore General Hospital (N = 60)	National University Hospital (N = 10)
Index case-patient			
Age (y)	22	60	64
Symptoms/diagnosis	Fever, headache, cough, patchy right infiltrate	Gastrointestinal bleeding, diabetic foot ulcer	Fever, increasing shortness of breath, hypotension
Time from admission to isolation (d)	6	10	1
Healthcare workers with probable SARS (%)[a]			
Doctors	8 (7)	3 (5)	1 (10)
Nurses	35 (32)	21 (35)	2 (20)
Ancillary caregivers[b]	3 (3)	6 (10)	0
Others[c]	3 (3)	10 (17)	0
No. of superspreading events	3 (including patient A)	1	1
Average time from onset to isolation (days)	4.6	2.6	1
Age (%)			
<20 y	10 (9)	0 (0)	1 (10)
20–29 y	40 (37)	7 (11)	4 (40)
30–39 y	25 (23)	13 (24)	1 (10)
40–49 y	11 (10)	10 (16)	0
50–59 y	15 (14)	11 (18)	0
≥60 y	8 (7)	19 (31)	4 (40)

[a]SARS, severe acute respiratory syndrome.
[b]Includes technicians, radiographers, and sonographers.
[c]Includes ward clerks, housekeepers, porters, and healthcare attendants.

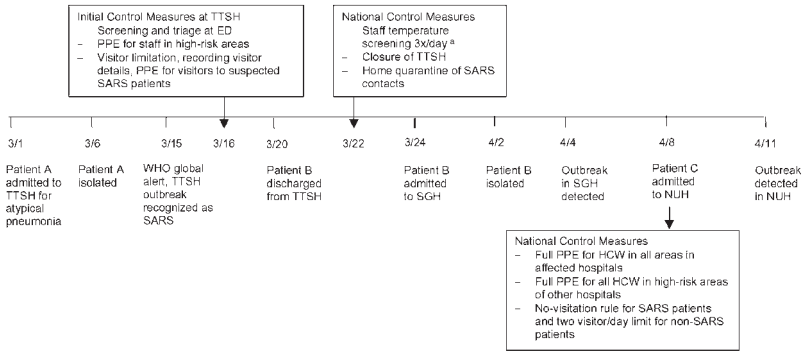

Figure 2. Timeline of events in the outbreak of SARS in the three acute hospitals, Singapore March–May 2003. SARS, severe acute respiratory syndrome; TTSH, Tan Tock Seng Hospital; ED, emergency department; PPE, personal protective equipment (defined as a test-fitted N95 mask, gowns, and gloves; goggles if dealing with suspicious cases; powered air purified respirators for high-risk procedures such as intubation); ICU, intensive care unit; high-risk area defined as ED, ICU, isolation wards; SGH, Singapore General Hospital; NUH, National University Hospital; HCW, healthcare worker. [a]Staff found to have a temperature >37.5°C were given medical leave for 3 days with a review on the third day.

from the CCU who had attended to patient AAA. In addition, TTSH was aware of five healthcare workers who were on medical leave for fever, four of whom were from ward 5A and one from ward 8A. Probable SARS subsequently developed in these five healthcare workers.

Probable SARS patients were isolated and their contacts were traced. Symptomatic contacts were isolated while asymptomatic contacts were advised to seek medical attention if they became ill. Infection control precautions were also enhanced in TTSH, including providing separate triage facilities for patients with suspected SARS who may seek treatment at emergency departments and requiring healthcare workers (in the emergency department, intensive-care unit [ICU], isolation wards, triage points, and wards which had been exposed to patients with possible SARS) to wear personal protective equipment consisting of N95 masks, gowns, and gloves. By March 21, approximately 30 probable SARS cases had been reported, of which half were healthcare workers. Persons in at least six wards and the CCU in TTSH had been exposed, but those in additional wards could have been exposed due to movements of infected healthcare workers. Therefore, on March 22, the decision was made to close TTSH's normal operations and dedicate the hospital solely to treating probable and suspected SARS case-patients (Figure 2). In line with this decision, all healthcare workers in the hospital adopted the use of N95 masks, gowns, and gloves at all times. A strict regimen (3x/day) of temperature surveillance of all staff was also instituted on March 21, with the aim of identifying affected staff as early as possible and isolating them immediately. The hospital limited visitors; visits to SARS case-patients were initially permitted, but visitors were required to use personal protective equipment (i.e., N95 masks, gowns, and gloves). Visitors to other ward areas were asked to use surgical masks. Non-SARS patients were discharged if they had no known exposure to SARS. Figure 2 summarizes the timeline of events in the outbreak and the key control measures instituted.

Outcome

The intrahospital transmission of SARS in TTSH was controlled within 3 weeks of instituting the measures (Figure 1). In retrospect, if more drastic measures had been implemented on March 15 (when SARS was recognized as the outbreak's cause), the containment of SARS infection in TTSH and Singapore could have been accelerated. In containing the TTSH outbreak, the inability to rapidly and completely identify all exposed persons was a major problem, mainly due to the absence of visitor records and the high frequency and complexity of patient-healthcare worker contacts and movements. Healthcare workers were also not using full personal protective equipment for most of that time.

If all contacts of the initial SARS case-patients detected on March 15 had been identified, patient AAA could have been cared for in isolation in CCU by staff with full personal protective equipment. At least 22 of the 27 case-patients infected by patient AAA in the CCU possibly could have been prevented.

The discharge of patient B from the same ward in TTSH as patient A was another factor which led infection to spread to SGH. Patient B had not been identified as a contact of patient A at that time and was subsequently readmitted to an open ward in SGH. In retrospect, we think that if all discharges from affected areas in TTSH had been stopped on March 15, the 60 probable SARS cases in SGH linked to patient B might have been prevented.

In-depth interviews to determine the epidemiologic link of SARS case-patients that arose after March 15 indicated that at least 17 of them visited TTSH areas where initial cases arose. If a no-visitor policy had been implemented in TTSH on March 15, at least 17 new SARS cases outside of TTSH could theoretically have been prevented.

SGH Cluster

The index case-patient in SGH was patient B, who did not exhibit typical signs and symptoms of SARS. Patient B was admitted to an open ward, ward 57, in SGH on March

24 for gastrointestinal bleeding. A fever attributed to an *Escherichia coli* urinary tract infection developed on March 26. On March 29, he was transferred to an adjacent open ward, ward 58, where he remained until April 2. He was transferred to the diagnostic radiology department twice, on April 1 and 2, respectively. Results of repeated chest x-rays were normal until shadowing in the right lower zone and left perihilar region was noted on April 5.

The SGH outbreak was identified on April 4 (Figure 2) when fever developed in a cluster of 13 healthcare workers. The cluster (one doctor, 11 nurses, and one radiographer) was detected because temperatures of all healthcare workers in SGH were monitored 3x/day. All 13 healthcare workers had attended to patient B; probable SARS developed in all 13. In total, the SGH outbreak resulted in 60 (24 healthcare workers, 11 inpatients, two outpatients, 12 visitors and 11 household contacts) probable SARS cases.

Public Health Response

The public health response was based on two key considerations. First, with 1,600 beds, SGH is the largest acute hospital in Singapore. With TTSH, the second largest acute hospital, dedicated as a SARS hospital, authorities could not shut down SGH as well. Second, the epidemiologic assessment was that the outbreak was localized. All SARS cases came from wards 57 and 58. Symptom onset for the 13 affected healthcare workers was March 31 for one case-patient, April 2 for three case-patients, April 3 for four case-patients, and April 4 for five case-patients. Most importantly, in line with SGH's standing rule, no affected healthcare workers came to work when they had fever. There was no evidence of secondary transmission.

The key containment strategy in the SGH was to completely remove exposed patients and healthcare workers to TTSH, the designated SARS hospital. Three main groups of patients were identified who might have been exposed to SARS in wards 57 and 58 during the "hot" period (i.e., when patient B was admitted on March 24 to the time his contacts were identified and transferred to TTSH on April 5).

The first group identified was the 80 patients in wards 57 and 58 on April 5. Next identified were a total of 135 patients admitted to the two affected wards during the hot period and subsequently transferred to three other wards in SGH. These three wards were subjected to a no-admission, no-discharge policy, and the two groups of patients were transferred to TTSH. The third group was 386 patients who had been in wards 57 and 58 in the hot period but had been discharged. None of these patients had fever, and all were called by phone 3x/day for 10 days.

Medical staff members in wards 57 and 58 were sent to TTSH to care for the transferred patients. An additional 236 SGH healthcare workers who had been in contact with exposed healthcare workers from the two wards were quarantined for 10 days.

Outcome

Containment measures controlled intrahospital infection spread within 10 days. Of the exposed patients and healthcare workers transferred to TTSH, probable SARS developed in eight. All were infected before the transfer.

However, two shortcomings affected the containment strategy. First was the failure to fully trace back the index case-patient's (patient B's) movement in the hospital. As a result, two exposed clinical areas were missed in the initial containment strategy. These areas were recognized when 11 additional cases were detected in two new areas in SGH: the diagnostic radiology department and a tertiary cancer facility, National Cancer Centre (NCC). Patient B had been in the diagnostic radiology department on April 1, where two healthcare assistants, two outpatients, and one visitor were infected. During patient B's second visit to the radiology department on April 2, an additional two healthcare workers who attended to him were infected. Fortunately, the cases were detected early because of SGH's strict 3x/day temperature monitoring regimen for all staff. In addition, all healthcare workers had begun using test-fitted N95 masks in all settings, including staff meetings and briefings. These steps helped mitigate further transmission of infection by healthcare workers. Unfortunately, one healthcare worker in the radiology area was given medical leave when she became febrile. She had close contact with some friends while on medical leave, leading to infection in three of them, one of whom infected four family members.

The second, more serious shortcoming arose because visitors to the two affected wards during the hot period were not completely traced. Patient B was visited by his brother, patient C, at SGH on March 31. Eight days after the visit, symptoms of SARS developed in patient C, who was admitted to an open ward in NUH.

NUH Cluster

Patient C sought treatment at the emergency department of NUH on April 8 (Figure 2). He remained in the emergency department for approximately 4 hours before being transferred to an open ward, ward 64, where he remained for about 8 hours before being intubated and transferred to the ICU. He remained isolated in the ICU until the morning of April 9 when he was transferred to TTSH; probable SARS was diagnosed after a complete history of contact was elicited.

Public Health Response

The NUH outbreak was identified on April 11 when a doctor who had attended to patient C was noted to have

fever. The key containment strategy in the NUH outbreak was isolating exposed persons (closing the exposed ward, stopping all new admissions and discharges to the ward for a period of 10 days, and isolating exposed patients). Staff members were put on work quarantine (i.e., they continued to work but were quarantined after work in separate quarters) and 3x/day temperature monitoring.

Outcome

As a result of these measures, when fever developed in two nurses 2 days after the outbreak, they were quickly recognized and isolated the same day. Fever developed in five inpatients in ward 64, who were transferred to TTSH. Two of these five inpatients had direct contact with patient B, and infections in the other three were likely acquired either from one of the infected healthcare workers or inpatients. The last probable SARS case from this ward was detected on April 25.

The shortcoming in this containment strategy was the failure to identify all the exposed visitors, one of whom had come into contact with patient C on April 8. This visitor was unidentified until probable SARS developed on April 11, by which time two family contacts had been infected.

Discussion

In the sequential SARS outbreaks, the three acute hospitals used different key containment strategies. On the basis of this experience, three key factors must be considered when deciding on the appropriate containment response. First, has the outbreak has been detected early? Second, can the likely source of the infection cluster be rapidly identified? Third, can a complete list of all contacts be obtained within 48 hours?

In our experience, if an outbreak is recognized early (within one incubation period and no evidence of secondary transmission on careful contact tracing) and the source identified and isolated expeditiously, the outbreak can probably be contained by closing the ward or clinical area, isolating all patients in the ward, and quarantining healthcare workers and visitors who have been in the ward. The NUH outbreak focused on this as its central containment strategy. The key was identifying and isolating patient C early. Patient C was in NUH for $\leq$12 hours before being isolated; hence, the number of exposed persons was manageable, and contacts could be identified with a high degree of confidence.

If, however, the outbreak is detected late (i.e., beyond 1–2 incubation periods, when secondary cases have occurred among staff and inpatients and multiple wards have been exposed), the most prudent course in our view is to close the hospital and place all healthcare workers on work quarantine as an immediate measure, while conducting epidemiologic investigations and implementing containment measures. We would generally consider an outbreak detected late if >1 maximum incubation period (10 days) elapsed before the outbreak was recognized. For example, the outbreak in TTSH was detected 14 days after the admission of patient A. In retrospect, by this time, secondary transmission in different parts of the hospital was indicated by the fact that SARS was diagnosed in a household contact of patient AA and a healthcare worker who attended to patient AAA in the CCU.

All patient admissions and discharges should be stopped. A complete list of all SARS patient contacts needs to be generated within 48 hours and those contacts quarantined or kept under medical surveillance, e.g., 3x/day telephone calls by a nurse. Otherwise SARS infection could rapidly spread to other healthcare facilities.

If closing the whole hospital is not possible, we believe that transferring all exposed persons to a designated SARS facility is the next best option. We have observed that running normal hospital services is difficult while managing a large number of patients with SARS or exposure to SARS. Scarcity of available beds may lead to transferring exposed patients prematurely out of isolation facilities into general wards, risking continued infection transmission. In addition, transferring all exposed persons out of the affected hospital allows it to carry on normal services as much as possible.

This containment strategy was adopted for the SGH outbreak and resulted in controlling intrahospital infection spread within 10 days. Movements of the index case-patient should be investigated to ensure that all exposed persons are quarantined. In the SGH outbreak, failing to identify the NCC and radiology departments as exposed areas led to missing exposed healthcare workers and patient contacts, and a small cluster of 11 secondary cases arose as a result.

Contact History

Patients' contact history must be reviewed carefully for contact with SARS patients. Because the contact history for both patients B and C was not thoroughly obtained at admission, they were not isolated, resulting in the SARS outbreaks in SGH and NUH, respectively.

Fever Surveillance of Healthcare Workers

In Singapore, all hospitals were required to establish a system of three times a day temperature surveillance of staff. In hospitals affected by SARS, temperatures were checked by designated staff and recorded. In other hospitals, staff could check and report their own temperatures. Temperature surveillance covered all healthcare workers, including healthcare attendants, cleaners, and contract staff who worked in clinical areas. Healthcare workers were not

allowed to work if their temperature was >37.5°C, and any healthcare worker with fever for >3 days or occurring as part of a cluster of cases was isolated. When more than two staff members or patients in a clinical area were febrile, epidemiologic investigation was initiated.

In our experience, fever surveillance among healthcare workers rapidly identified potentially infected healthcare workers in hospitals with SARS outbreaks. In the SGH outbreak, fever surveillance enabled the rapid detection of 7 of the healthcare worker probable cases that arose from the NCC and radiology departments. Similarly in the NUH outbreak, temperature surveillance identified two ward 64 nurses who were isolated the same day.

Personal Protective Equipment

On the basis of our and others' experiences (5), the nonspecific symptoms of SARS make identifying potential cases difficult. Strictly adhering to the use of personal protective equipment by healthcare workers mitigated spread in all three outbreaks.

Visitor Records

In all three hospital outbreaks, visitors contributed substantially to SARS transmission. At least 21 cases resulted from spread by hospital visitors to family and community contacts. This type of transmission emphasizes the importance of maintaining a visitor log and limiting visitors. In Singapore, a no-visitor policy in all public hospitals was implemented 2 weeks after detecting the NUH outbreak. Similar restrictions on hospital visitors were implemented as control measures in outbreaks elsewhere (5).

Better Preparedness

The outbreak in TTSH was central to spreading infection to the other two hospitals. Key difficulties in containing the TTSH outbreak were: 1) late recognition of disease, 2) lack of understanding of the disease, 3) inadequate infrastructure to support outbreak management of this scale, 4) lack of ability to identify atypical cases, and 5) lack of understanding of superspreading events.

Some of these difficulties can be overcome by better planning and preparedness. However, a large part of an effective response will rely on thoroughly understanding the disease. In Singapore, we tried to develop a better response capability through several means. The first component was prevention through the use of N95 masks, gowns, and gloves by healthcare workers in high-risk clinical areas and triaging febrile patients at emergency departments and outpatient clinics, followed by isolating infectious patients early. The second component was detecting possible SARS clusters early through surveillance for clusters of febrile healthcare workers or patients. The third component was ensuring that all hospitals have established and tested systems to rapidly generate a complete list of all potential healthcare workers, patients, and visitor contacts. These components should be part of a hospital preparedness plan.

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The RUsick2 Foodborne Disease Forum for Syndromic Surveillance

Holly Wethington* and Paul Bartlett*

The RUsick2 Foodborne Disease Forum at the National Food Safety and Toxicology Center increased reporting of foodborne diseases to more than four times the rate seen in the previous 2 years. Since November 2002, the Forum has allowed pilot-area residents with sudden-onset vomiting or diarrhea to share and compare information regarding what they ate and did before becoming sick. The purpose is to identify a common food source, perhaps resulting in identifying a cluster of persons who ate the same contaminated food item. Such information can assist health departments in detecting foodborne outbreaks while the possibility for intervention remains.

Foodborne infection is the cause of approximately 76 million gastrointestinal illnesses and approximately 5,000 deaths each year in the United States (1), but causes are rarely identified. Nationally, an estimated 1%–2% of cases are reported annually (2). Given that two or three reported cases are required to recognize and define a common source outbreak, many small- and moderate-sized outbreaks escape detection. Table 1 depicts the results of a binomial analysis of outbreaks of various sizes, given an assumed 2% reporting. For example, with 2% reporting, an outbreak of 75 cases would have a 44% chance of having two or more cases reported and a 19% chance of having three or more cases reported. Many outbreaks are never detected, and many reported cases seen as sporadic and isolated may in fact be part of small, undetected outbreaks.

Current laboratory-based surveillance will likely not substantially increase the percentage of routine gastroenteritis cases that provide samples for culturing. Health insurance organizations are not expected to increase the numbers of fecal samples submitted and cultured for uncomplicated cases of gastroenteritis, since culture results do not usually influence the medical management of individual cases.

A second problem is the time delay inherent in current laboratory-based passive surveillance. On the basis of a 2000 survey of reported cases of foodborne illness, 263 Michigan hospital laboratories (response rate 91%) averaged a delay of 12.3 days between specimen collection and serotyping. In addition, a mean delay of 35 days occurred between symptom onset and completion of the case investigation form by the local health department (Michigan Department of Community Health, unpub. data). Given the short duration of most foodborne outbreaks, health department investigations are often a matter of documenting past events, with no real opportunity to quickly identify and remove contaminated food items to prevent further exposure.

A third constraint of current surveillance is that it is based almost entirely on paper forms or individual telephone reports to local health departments. This system can manage sporadic cases and small outbreaks, but larger outbreaks would quickly overrun the capacity of most local health departments. The inability to adequately investigate large outbreaks is especially important given the potential for intentional contamination of food supplies as an act of bioterrorism or biowarfare.

Examining these three limitations evolved into a plan to implement a syndromic surveillance forum in which clusters of foodborne disease could be quickly identified for further investigation. This system would act as a method to augment existing laboratory-based surveillance and would identify clusters of persons with suspected foodborne disease that warrant further investigation by health departments.

The RUsick2 Forum

The RUsick2 Forum was developed by epidemiologists from the Michigan Department of Community Health, the Michigan Department of Agriculture, the University of Michigan, Lansing-area health departments, and Michigan State University. All were brought together under the auspices of the National Food Safety and Toxicology Center at Michigan State University.

Data Input

A Web site (www.RUsick2.msu.edu) was developed to record information on symptoms, time of illness onset, a

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Table 1. Simulated binomial data, assuming 2% of cases reported

Size of outbreak	2 or more reports	3 or more reports
25	9%	1%
50	26%	8%
75	44%	19%

4-day preillness food history, food sources, and other information regarding nonfoodborne sources of common gastrointestinal illness. Visitors can potentially view 22 screens, most of which are data input screens with a few displaying other visitors' data (no personal identifiers are viewed by RUsick2 visitors). The Forum allows visitors to return multiple times to modify their data if they recall more about what food they consumed and where they purchased it. The Centers for Disease Control and Prevention (CDC) Standard Foodborne Questionnaire and other foodborne questionnaires were emulated in creating the data input screens (3). The food list currently contains 54 food items, divided into the following categories: popular main courses, meats/poultry/fish, dairy and eggs, raw fruit, raw vegetables, prepared fruit or vegetables, salad items/side dishes, grains and starches, and beverages. Figure 1 displays an abbreviated version of this data entry screen.

A section concerning nonfood exposures was incorporated to gather information regarding exposure to animals, sick persons, patients in a healthcare setting, commercial food preparation, young children, private well water, and swimming (lake/river or swimming pool). The Forum is unlike most Web-based forums in that it is structured and does not allow narrative testimony. As with written or telephone reporting, persons generally have a difficult time remembering what they consumed during the several days before becoming ill. Computer technology does not enhance memory, but it allows the reporter to recall data at his own pace and return to the Web site to add or modify data after consulting friends, family, calendars, checkbooks, and credit-card records.

A follow-up survey is being conducted of all visitors to the Web site. Virtually all modifications to the program instituted after November 2002 shortened and simplified the program. We intend to continue modifying the program to meet the requests of RUsick2 visitors. Moreover, focus groups are planned for the future to further increase usability.

Information Retrieval

As the visitor proceeds through the program entering data regarding symptoms, food items eaten, and food sources, increasingly specific comparisons with other users' data are available. The objective is to help each visitor determine what he might have in common with other persons, including symptoms, time of illness onset, and consumption of the same food item from the same food source.

The summary report is a univariate descriptive analysis showing the number and percentage of past visitors who reported the same risk factors (foods, food sources, nonfood exposures) as the current visitor, who can use the summary report to select individual reports for viewing. A comparison report analyzes the visitor's risk factors during an adjustable "target" period of onset dates, compared to an adjustable historic "comparison" period. Subsequent retrievals can be restricted on the basis of risk factors, and data can be viewed as a case report or output in a format accessible by most spreadsheet, database, and statistical programs. Figure 2 shows an example of the comparison report.

Visitors may choose to enter their data and leave the investigation to the health departments to determine whether a cluster exists. They may also view a descriptive table of recent reports and see whether the source they originally suspected was mentioned by other Forum visitors. Persons who are satisfied that they are not part of an outbreak may leave the Forum without requesting further output. Those who see common exposures may pursue more sophisticated output. At any time in the process, Forum visitors can request the aid of their health department.

The Forum does not investigate outbreaks or replace the current systems used by local health departments. Rather, the Forum increases reporting of foodborne illness and makes identifying suspicious clusters that may warrant further investigation possible. By collecting numerous variables, the Forum delivers information on a large number of risk factors to local health departments to assist in an investigation.

A moderator views each report after it is entered and conducts appropriate follow-up. For example, if a report from outside the three-county pilot area is entered, the moderator will alert the proper health department that a report from their jurisdiction has been entered. Like most

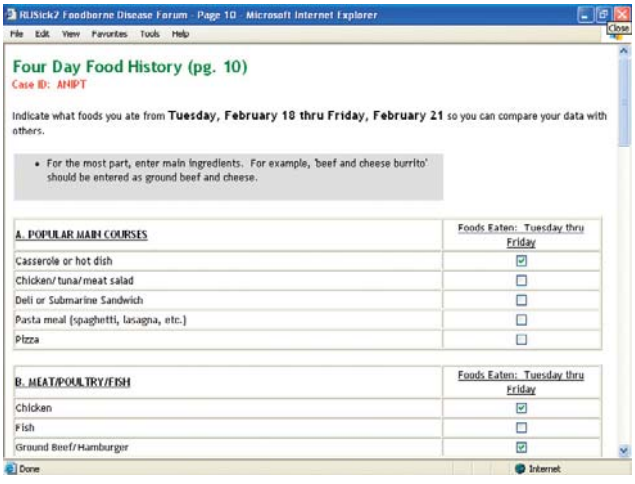

Figure 1. Abbreviated food history data entry screen.

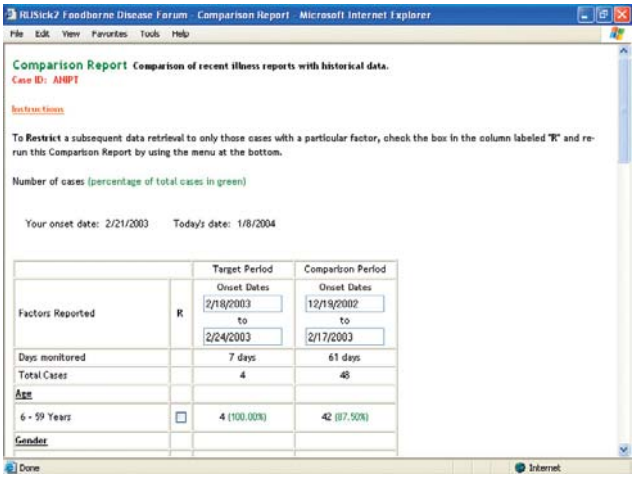

Figure 2. Comparison report displays each risk factor the visitor reported during his target period along with a comparison period.

Web-based forums, the moderator also reviews data for reasonableness, profanity, or other infringements of posted Forum rules. Records can be excluded from analysis, and each health department may similarly reject records.

Health Departments

RUsick2 visitors cannot retrieve other visitors' personal identifiers, narrative testimony, or names of restaurants, stores, or other food sources. In contrast, health departments have access to all data fields for visitors who report being residents of their jurisdiction. Local health departments in the three-county pilot area around Lansing, Michigan, were involved in developing the Forum and have helped design methods by which local health departments can monitor the Forum. During the pilot phase, local health departments have had password access to the database to check reports from their residents. After expansion of the Forum, counties with less activity will be able to request automatic email notification from the Forum's computer.

Phantom Outbreaks

Several features of the Forum are designed to prevent phantom outbreaks caused by the power of suggestion. Visitors cannot implicate any particular food item or exposure. Second, visitors enter data before being given the opportunity to view output that might influence their own reports. (While visitors may modify their responses on subsequent visits to the Forum, very few do.) Third, each visitor only views output for food sources, foods, and other risk factors they have already indicated in earlier data entry screens. Fourth, food stores, restaurants, and other food sources are identified to Forum visitors only by abbreviations, which may not be specific to particular establishments. Abbreviations are sufficient to identify suspicious clusters, but only health departments see the entire names of commercial establishments. Finally, suspicious clusters must be investigated by the local health department to determine if clusters are due to foodborne outbreaks, chance, confounding, pranks, or normal changes in diet.

Pilot Test

The Forum is being pilot tested in the tri-county area of Clinton, Eaton, and Ingham counties, which make up the Greater Lansing, Michigan area. The Forum was implemented in November 2002, involving three local health departments: the Barry-Eaton District Health Department, the Ingham County Health Department, and the Mid-Michigan District Health Department. For comparison purposes, we evaluated previous foodborne illness reports from the population of these counties for the years 2000 and 2001 (4–6).

Publicity

The target population was 450,000 residents living in the three pilot counties. The percentage of this population that has Internet access is unknown, but an estimated 51.2% of all Michigan households had Internet access in 2001 (7). This percentage does not include persons who may have access at work, school, or public libraries.

Advertisements were published five times a week in the area's daily newspaper (The Lansing State Journal). Fliers and brochures were distributed to 450 Lansing-area physicians in October 2002 with a letter explaining the project. Local television channels featured the project on various newscasts. In addition, three newspaper articles about the project appeared in the daily newspaper (8–10), two articles appeared in the university's independent student newspaper (11,12), and one article ran in a smaller weekly paper (a subsidiary of the area's largest newspaper) and a local township paper (13,14). Articles have been printed in various health departments' newsletters and other university-related publications.

A student employee worked 2 days per week visiting private physicians' offices, urgent care offices, emergency rooms, and pharmacies to distribute fliers and brochures and to ask clinic nurses to recommend the Forum to patients with suspected foodborne illness. The Forum has been described at local grand-rounds meetings for internal medicine, emergency medicine, pediatrics, and family practice.

Publicity given to this project may have influenced the results. The Forum was well advertised in the three-county pilot area. Reports could have increased as a result of advertising, regardless of mode of reporting. However, one local health department involved in the pilot project stated that the number of traditional reports had not increased since the outset of the RUsick2 Forum.

Illness Reporting

Table 2 depicts the Michigan Department of Agriculture data collected for 2000 and 2001 by year, month, and county. From these data, we predicted that approximately 22 reports would have been expected during the comparable months of 2002 and 2003. The RUsick2 data are displayed by month and county in Table 3 and show that 93 reports were obtained with the Web-based system.

From November 2002 (the time the Forum was implemented) until February 2003, a total of 93 reports to the RUsick2 Forum reached at least the entry level of reporting (which begins by identifying foods consumed). In the first 17 weeks of the program, an average of 5.37 cases were reported each week. Based on the previous years' reports, we calculated an expected number of 1.31 cases per week; hence, the ratio of reported cases to expected reports (5.37/1.31) was 4.10.

Figure 3 shows the weekly average of foodborne complaints reported to the Forum from the three pilot counties during the first 17 weeks of operation. Also shown on this graph is the weekly average number of foodborne disease complaints reported to the state of Michigan during the corresponding months of January, February, November, and December 2000–2001. Approximately 22 reports would have been expected during the 17-week period, based on reports from previous years. However, 93 reports were received during the first 17 weeks of operation, more than a fourfold increase in reporting.

Local health departments monitored reports to the Forum and contacted reporting persons by telephone or email to verify the accuracy of the report and authenticate the complaint. Contacts usually required a few minutes. Reports delivered from the Forum to local health departments were treated as traditional reports. The Forum identified two foodborne outbreaks that would likely not have been identified. One fictitious report was identified and rejected.

Future Development

Similar to other Web-based forums, RUsick2 allows persons with a common health problem to examine one another's information. In an attempt to determine if they share any common exposures, visitors can view risk factors such as food histories, food sources, and other exposures entered by previous visitors. The Rusick2 Forum acts as a "front end" to existing surveillance by increasing reporting and identifying suspicious clusters that warrant a full investigation.

The Forum has recently been adapted for national usage so that the technical aspects of data entry and information retrieval will function identically for residents of all states. The publicity campaign conducted in the pilot counties will be too expensive to reproduce on a national level, so the Forum will only gain national prominence if local health departments and consumer advocacy groups use and publicize the Web site. The input screens of this program are easily modifiable or removable. For example, the

Table 2. Foodborne disease reports in Michigan by county, month, and year[a]

County	Y	Jan	Feb	Nov	Dec
Ingham	2000	9	4	4	3
	2001	1	2	0	4
Clinton	2000	0	0	0	0
	2001	0	0	0	0
Eaton	2000	1	2	3	1
	2001	4	4	1	2
Total		15	12	8	10
No. wk/m		4.42	4.00	4.29	4.42
2000–2001 average/wk		1.70	1.50	0.93	1.13

[a]Source: Michigan Department of Agriculture, unpub. data.

Table 3. RUsick2 visits by county, month, and year

County	Y	Nov	Dec	Jan	Feb
Ingham	2002	15	13	--	--
	2003	--	--	25	9
Clinton	2002	0	1	--	--
	2003	--	--	1	3
Eaton	2002	7	7	--	--
	2003	--	--	12	3
Total		22	21	38	12
No. wk/m		4.29	4.42	4.42	4.00
Average/wk		5.13	4.75	8.60	3.00

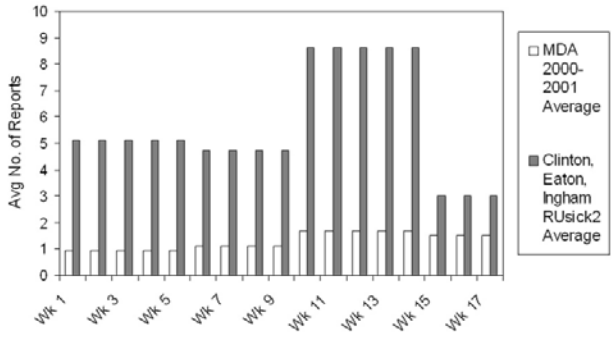

Figure 3. Comparison of foodborne disease reports from Ingham, Eaton, and Clinton Counties before and after implementing the RUsick2 Forum; MDA, Michigan Department of Agriculture.

symptoms screen can be altered to include symptoms specific to a non-foodborne disease, or the food history screens can be deleted entirely. Thus, this program could be adapted for use in other disease outbreaks.

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Reemerging Leptospirosis, California

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Leptospirosis is a reemerging infectious disease in California. Leptospirosis is the most widespread zoonosis throughout the world, though it is infrequently diagnosed in the continental United States. From 1982 to 2001, most reported California cases occurred in previously healthy young adult white men after recreational exposures to contaminated freshwater. We report five recent cases of human leptospirosis acquired in California, including the first documented common-source outbreak of human leptospirosis acquired in this state, and describe the subsequent environmental investigation. Salient features in the California cases include high fever with uniform renal impairment and mild hepatitis. Because leptospirosis can progress rapidly if untreated, this reemerging infection deserves consideration in febrile patients with a history of recreational freshwater exposure, even in states with a low reported incidence of infection.

Leptospirosis was distinguished and described in the early 18th century by Adolf Weil and several other scientists, although several references to epidemic jaundice and bilious typhoid likely related to leptospirosis appeared in many ancient cultures. The syndrome's many colorful names include "rice-harvest jaundice" in China, "autumn fever" in Japan, "swineherd's disease" in Europe, and "sewerman's flu" in the United States (1). Leptospirosis is a reemerging infectious disease, involving both rural and urban cases (2–6).

Leptospira are transmitted in the urine of chronically infected carrier animals. Numerous serovars of pathogenic *Leptospira interrogans* are known to infect humans. Rats are universal reservoirs for this spirochetal zoonosis, although farm animals and livestock can also harbor the infection. In disease-endemic areas, cattle are vaccinated against leptospirosis. Infection occurs when spirochetes in contaminated water or soil enter microabrasions on the skin or intact mucous membranes (1–3,7). The incubation period is approximately 10 days (range 4–19 days) (8). In temperate climates, peak incidence is during the summer, when leptospires survive longer in the environment (3), and water exposures may be more common.

Leptospirosis is an acute febrile illness with nonspecific clinical signs and symptoms and a variable clinical course. Clinical manifestations include fever, malaise, myalgia, meningism, and conjunctivitis, as well as anorexia, abdominal pain, nausea, and vomiting (1–3,7). Initial signs of serious infection can include jaundice, hemorrhage, and hepatosplenomegaly. The most severe form of leptospirosis can occur as Weil syndrome with hepatic and renal failure or massive pulmonary hemorrhage; both forms can progress rapidly to death if untreated (1–3,9).

Leptospirosis is thought to be the most widespread zoonotic disease in the world (10). Most reported cases occur in men, most likely due to greater occupational exposure (11). Recent outbreaks associated with water sports and recreation include 68 of 304 athletes participating in a 2-week adventure sport race, the Eco-Challenge-Sabah 2000, in Borneo, Malaysia (12), and 74 of 639 triathletes in 1998 races in Wisconsin and Illinois, in the largest known outbreak of leptospirosis in the United States (13).

Leptospirosis in the United States

The reported incidence of leptospirosis is 100–200 cases per year in the United States (2,14), with most (50–100 cases) occurring outside the continental United States in Hawaii (15). Leptospirosis is likely underdiagnosed in the United States, with reported incidence depending largely upon clinical index of suspicion (3,6). Historically, workers in direct contact with animal reservoirs—especially cattle and pig farmers, slaughterhouse workers, veterinarians, and dairy farmers—have been considered to be at increased risk. Recreational exposure may occur from swimming or boating in freshwater lakes formed by runoff or damming (1). Infections have been reported in HIV patients (16) as well as children (17), and urban residential exposure is on the rise, most notably in

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crowded inner city locations with rat infestations (5). Sporadic outbreaks of leptospirosis in the continental United States have occurred in the East, Midwest, and in Texas in the last decade (5,13,18)

Leptospirosis in California

Leptospirosis has been a reportable disease in California since 1922, even after its removal from the national reportable disease list in 1995, and completion of a case history report form has been required since 1966 (19,20). In the 20-year period 1982 to 2001, 61 cases of leptospirosis were reported to the California Department of Health Services, for an average of 2.8 cases per year: most of these were imported by vacationers to Hawaii, Malaysia, and other tropical locales outside the state. However, more than half of these cases occurred in the most recent 5 years alone; in the 5-year period 1997–2001, 34 cases were reported, for an average of 6.8 cases of leptospirosis per year (21). Thus, the overall incidence of leptospirosis in California appears to be on the rise.

In-state acquisition of leptospirosis in California shows a similar trend. In the 20-year period 1982–2001, 23 reported cases of California leptospirosis were also acquired within California, for an average of 1.15 in-state cases per year. Twelve of these cases occurred in the most recent 5 years, for an average of 2.4 in-state cases per year (21). During this period, the population of California rose from 24 million to an estimated 34 million (22).

Several trends in the epidemiology of leptospirosis in California are apparent over the last 20 years. Most reported cases appeared in previously healthy adult white men after a recreational exposure to contaminated freshwater. Of the 61 California cases in the last 20 years, at least 77% occurred in men. At least 67% occurred in adults of ages of 20 to 40 years, and at least 70% occurred in persons identifying their ethnicity as white. Recreational exposures accounted for 59% of cases, 16% were occupational, 10% were pet-related, and 15% were due to unidentified exposures. Recreational exposures may be on the rise, as they accounted for a full 85% of exposures in the 34 cases in the most recent 5 years (Table 1) (21).

These patterns are also illustrated by the five cases of leptospirosis acquired within the state in the past 3 years (Figure 1). In 1999, there was one sporadic case associated with exposure to freshwater while the patient was duck hunting in Butte County, or possibly following contact with rodent urine in an infested trailer. In 2000, three cases were associated with an outbreak among San Mateo County residents who swam in a reservoir in Tuolomne County while on a houseboat vacation. In 2001, one sporadic case was reported in a woman from Santa Clara County associated with swimming in a muddy pond. We describe these five cases, including the first documented common-source outbreak of leptospirosis acquired in California.

Table 1. Features of leptospirosis patients reported in California, 1982–2001

Exposure type	% (n=61)
Recreational	59
Occupational	16
Pet-related	10
Unknown	15
Sex	
Male	77
Female	15
Unknown	8
Race	
White	70
Hispanic	8
Black	2
Unknown	20
Age	
0–20	10
21–40	67
41–60	13
60+	2
Unknown	8

Diagnosis of Leptospirosis

The diagnosis of leptospirosis can be made by use of rapid serologic assays. The microagglutination test (MAT) has been the reference method for serologic diagnosis of

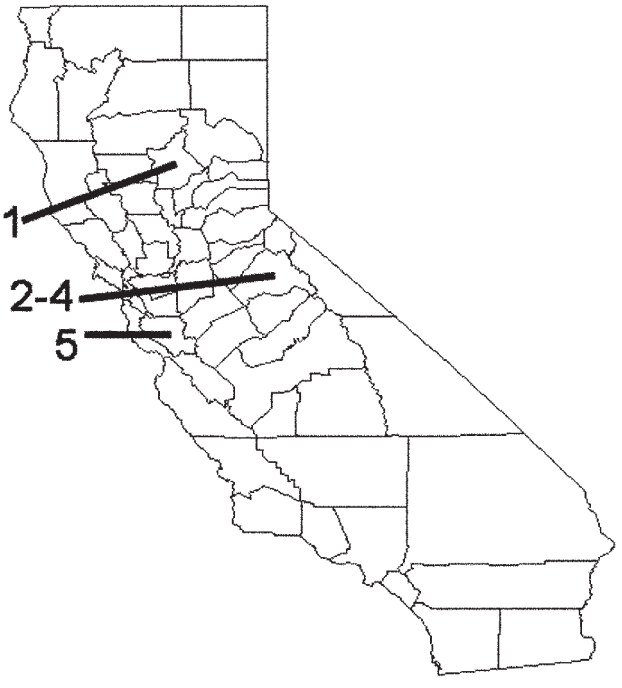

Figure 1. California state map showing case report exposures by county: Case 1, Butte County; cases 2-4, Tuolomne County; case 5, Santa Clara County.

leptospirosis. MAT utilizes antigens from serovars representative of all serogroups, since cross-reactivity between serovars occurs frequently. Though a titer of >1:400 may be considered confirmatory in the appropriate clinical context, a fourfold or greater rise in titer over any time period confirms the diagnosis. A second test is the indirect hemagluttination assay (IHA) for both immunoglobulin (Ig) M and IgG antibodies. A third assay is an enzyme-linked dot immunoassay for IgM antibodies in serum (IgM-Enzyme-Linked ImmunoSorbent Assay [ELISA], the Dip-S-Ticks [Integrated Diagnostics, Inc., Baltimore, Maryland), that appears to have a greater sensitivity early in infection than the other available assays. The IgM-ELISA is more readily available and is less labor-intensive than MAT, especially when paired samples for serologic testing are unavailable. However, the result of IgM-ELISA should be considered preliminary and further confirmation by MAT is recommended. In addition to the above serologic assays, polymerase chain reaction and immunohistochemical (IHC) assays are sensitive microscopic methods of diagnosis if tissue sample is available for testing (3,6).

Case Reports

1999: Case 1

In mid-January, a 38-year-old man went duck hunting in flooded rice fields in Butte County; he also stayed in a trailer that had stood vacant for some time, where he recalled cleaning up rodent droppings. On January 27, jaundice, pulmonary infiltrates, and renal failure developed. Laboratory studies showed bilirubin of 48 mg/dL and platelets of 13,000/μL.

Serologic testing for hantavirus was negative. IHA for leptospirosis at a private laboratory 9 days later yielded an initially ambiguous titer of 1:50, and a second test 11 days after that yielded a titer of 1:800 (Table 2), greater than the fourfold rise required to confirm the diagnosis. He was treated successfully with doxycycline.

2000: Cases 2–4

In 2000, the largest known common-source outbreak of leptospirosis in California involved three men vacationing on a houseboat in the New Melones Lake in Tuolomne County May 5–7. Three of eight men who shared the houseboat reported swimming to a remote cove and hiking along a creek draining into the main reservoir on May 7. They were exposed there to muddied waters after an overnight thundershower. Each of these three men went individually to his own doctor and independent health system in Redwood City 10–15 days later, with a constellation of signs and symptoms including fever, headache, myalgias, nausea, and vomiting. In each, the salient common clinical feature of renal failure developed, manifested by elevated creatinine levels that in one case required hemodialysis.

Case 2

A 35-year-old Caucasian man went to an urgent care clinic twice before being admitted to the hospital with fever as high as 39.4°C, myalgias, and renal insufficiency later. Additional symptoms included headache, photophobia, nausea, and anorexia, starting 10 days after returning from the boating trip. Signs included subtle diffuse flushing and conjunctival suffusions. Initial laboratory values showed a mild hepatitis with a rising creatinine that increased daily to peak at 4.0 mg/dL. Liver enzymes were elevated with peak values of alanine aminotransferase (ALT) 258 U/L, alkaline phosphatase 189 U/L, and bilirubin 3.5 mg/dL. Results of examination of the cerebrospinal fluid were normal. Leukocyte count was within normal range at 4.7 x 10^9/L. By the third day of hospitalization, after IV rehydration and broad-spectrum antimicrobial agents, the patient's fever subsided with improvement of his myalgias and headache. He was discharged and given doxycyline, 100 mg twice daily for 10 days. He had no clinical sequelae.

Serologic tests for infectious hepatitis A, B, C, and brucella, and a Monospot test were negative. MAT and IgM-ELISA for leptospirosis were processed at the Centers for Disease Control and Prevention (CDC), and the California Department of Health Services (CDHS), respectively. Titers from serum drawn 15 days after the initial exposure were negative; however, convalescent-phase serum drawn 17 days later tested positive for leptospira by IgM-ELISA

Table 2. Acute- and convalescent-phase serologic and microscopy diagnosis of leptospirosis by MAT,[a] IgM-ELISA, IHA, and IHC assay in California case reports[b]

Case	Case 1		Case 2		Case 3		Case 4		Case 5	
Days post exposure	9	11	15	32	21	36	23	44	Acute	Conv
MAT	ND	ND	Neg	1:3200	Neg	1:800	1:800	1:200	Neg	ND
ELISA	ND	ND	Neg	Pos	Pos	ND	Pos	ND	Pos	ND
IHA	1:50	1:800	ND	ND	ND	ND	ND	ND	ND	1:200
IHC assay	NA		NA		NA		Pos		Pos	

[a]MAT, microagglutination test. Highest MAT titer reported to any leptospira serogroup.
[b]IgM-ELISA, immunoglobulin M–enzyme-linked immunosorbent assay; IHA, indirect hemagluttination assay; IHC, immunohistochemical; NA, not available; ND, not done; Conv, convalescent phase.

at CDHS, and MAT at CDC showed the strongest reaction with serovars from *L. interrogans* serogroups Australis and Mini (Table 2).

Case 3

A 35-year-old Caucasian man went to a hospital emergency department 15 days after returning from a houseboating trip to New Melones Reservoir in the Sierra Nevada foothills of northern California. He reported a 4-day history of headache, fatigue, low back pain, myalgia especially of the calves, vomiting, and fever as high as 40.6°C. Signs included erythema of the left flank, with scattered tiny red macules on the legs. Initial laboratory studies showed proteinuria, a creatinine level that peaked at 1.7 mg/dL, and mild transaminasemia, with ALT 182 U/L and alkaline phosphatase 370 U/L. His leukocyte count was within normal range at 9.0 x 10^9/L.

Other serologic tests, including for hepatitis A, B, and C, rickettsia, brucella, and ehrlichia, were all negative. He took doxycycline, 100 mg orally twice daily for 10 days, and made a complete recovery. MAT and IgM-ELISA for leptospirosis were processed at CDC; titers from serum taken 21 days and 36 days after the houseboat trip were most strongly positive with serovars from *L. interrogans* serogroup Mini (Table 2).

Case 4

A 38-year-old man went to the hospital emergency department 13 days after the houseboat excursion, with fever of 37.8°C, tea-colored urine, myalgias, chills, headache, anorexia, nausea, and abdominal pain. He was admitted to the hospital with oliguric acute renal failure. Peak elevated laboratory values included a creatinine of 17.3 mg/dL, blood urea nitrogen 82 mg/dL, urobilinogen 4+ EU/dL, and urine protein 100 mg/dL. His leukocyte count was within normal range at 5.6 x 10^9/L.

Serologic tests for hepatitis A, B, C, rickettsiae, and ehrlichiae were negative. Renal biopsy 17 days post exposure showed acute tubulointerstitial nephritis (Figure 2A), which is reported to be the most characteristic renal lesion observed in leptospirosis (23,24). Ultrasonography showed bilateral pleural effusions and echogenic kidneys. He received broad-spectrum antimicrobials and dialysis and remained hospitalized for almost a month. His acute renal failure resolved, and he made a full recovery. MAT and IgM-ELISA results for leptospirosis were processed at CDC; titers from serum taken 23 days and 44 days postexposure were most strongly positive with serovars from *L. interrogans* serogroup Mini (Table 2). Immunohistochemical assay for leptospira was performed at CDC and showed granular immunostaining of leptospira in the kidney biopsy.

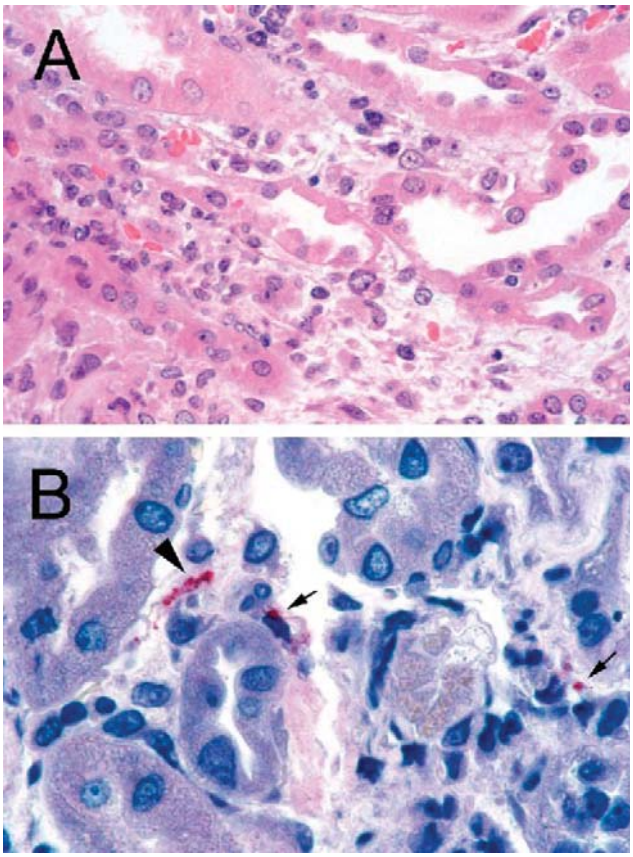

Figure 2. A: Renal biopsy shows inflammatory cell infiltrate in the interstitium and focal denudation of tubular epithelial cells. Hematoxylin and eosin; original magnifications x100. B: Immunostaining of fragmented leptospire (arrowhead) and granular form of bacterial antigens (arrows). Original magnifications x158.

2001: Case 5

A 53-year-old woman went hiking in the foothills of Mount Hamilton in Santa Clara County on September 1, 2001, where she swam in a muddy pond. Eleven days later, she had the onset of shaking chills, fever of 38.9°C, myalgias, ankle and wrist pain, and headache. Her fever resolved, but 4 days later nausea, vomiting, diarrhea, and a worsening headache developed and she went to urgent care. Initial laboratory test results including cerebrospinal fluid, blood urea nitrogen, and creatinine were normal, and she was treated for gastroenteritis with promethazine HC1 and fluids.

Three days later, oliguria developed. On September 19, she was admitted with oliguric renal failure and signs including an erythematous macular rash on her face and pretibial macular blanching lesions on her legs. Over the next few days, a persistent cough with rales developed, and peak levels of blood urea nitrogen were 101 and creatinine were 9.5. Urinalysis showed 1+ blood, 6–10 erythrocytes, 0–3 leukocytes, and 3+ protein. Though her leukocytes were normal at 5.9 x 10^9/L with 84% polymorphonuclear

leukocytes, her platelet count was low at 122,000. Mild transaminasemia also developed as well as an elevated International Normalized Ratio. She was treated with high-dose penicillin G and corticosteroids.

A renal biopsy, which showed tubulointerstitial inflammation, was performed. Immunohistochemical assays for several organisms were performed at CDC; the results were negative for hantavirus and spotted fever group rickettsiae, while immunohistochemical assay for leptospira showed granular immunostaining in the renal biopsy to confirm the diagnosis of leptospirosis (Figure 2B). The patient was discharged after 6 days on doxycycline 100 mg twice daily for 10 days and made a full recovery. Acute-phase serum was positive for leptospira IgM antibody by ELISA but negative for all serovars by microagglutination test at CDC. Convalescent-phase serum was positive for Leptospira by indirect hemagluttination assay at a private laboratory (Table 2).

Environmental Investigation

The treating physicians in the 2000 New Melones outbreak initiated a public health inquiry to investigate potential environmental risks. This investigation identified potential sources of infection but did not identify a definitive source.

Five weeks after the New Melones exposures, a multidisciplinary team traveled to the reservoir to sample environmental sources for leptospires and to direct potential public health interventions. The physician for the first case, the state public health veterinarian, environmental health researchers from the University of California at Berkeley, and the local environmental health and sheriff's departments collaborated to better define the origins of this disease outbreak.

The team documented cattle herds grazing in the region above Wolf Gulch, where the three patients were presumed to have been infected. Leptospirosis is commonly transmitted following flooding that creates standing water in regions where cows or other animals graze (1–3,25). Though wildlife, including rodents, ungulates, and small carnivores, is common in this area, the proximity of the cattle and evidence that leptospirosis vaccination does not prevent shedding by cattle implicate these herds in the transmission of infection. However, the cattle were not tested for leptospiral antibodies because of their likely vaccination history. Furthermore, New Melones is a lake created by a dam of the Stanislaus River, and runoff between April and July is usually from snowmelt. After a warm, dry period of almost 3 weeks with temperature maximums in the high 70s and 80s, rain fell on May 6 and 7 totaling less than 1/2 inch (26). This weather shift could have provided the mechanism for contaminated animal urine to drain into the creeks surrounding the reservoir.

Using the Moore swab technique for water collection, the team collected 5 gallons of water from three sites in the reservoir, filtering them through sterile cotton plugs which were then incubated in leptospiral media (Ellinghausen McCullough Johnson Harris [EMJH] medium) at 29°C for several days. Though two of the three samples were positive for spirochetes upon darkfield microscopy, they were negative by polymerase chain reaction using primers specific for pathogenic strains of leptospira, a result of ambiguous significance in this case.

Once the working diagnosis of leptospirosis was formulated, the county public health departments were notified where the cases were reported (San Mateo County) and where the exposures occurred (Tuolomne County). Public health notices warned primary caregivers and emergency departments in these areas to consider leptospirosis in patients with fever, headache, and myalgia who had been exposed to the water of Lake Melones. The Tuolomne General Hospital emergency department identified a potential fourth case of leptospirosis on June 6, in a 23-year-old man with headache, myalgias, chills, neck pain, and vomiting, 5 days after swimming in New Melones Lake; symptoms resolved with doxycycline, and leptospirosis titers were not obtained.

Common Clinical Features

Salient features in the California cases include high fever with uniform renal impairment and mild hepatitis. Further clinical features shared in all five cases of leptospirosis in California include headache, nausea/vomiting, and myalgia. All five patients had elevated creatinine levels and normal leukocyte counts. All patients were treated with doxycycline and made complete recoveries after variable levels of severity in the course of their illnesses.

Discussion

Leptospirosis is a reemerging infection in California, with most cases appearing in young adult white men after recreational freshwater exposures. This report includes the first documented common-source outbreak of human leptospirosis in California.

In addition to leptospirosis, the differential diagnosis for a patient with fever, fatigue, stiff neck, headache, nausea and vomiting, and myalgias should include mononucleosis, hepatitis viruses B and C, meningitis, and zoonotic infections such as brucellosis, tularemia, hantavirus, dengue, Colorado tick fever, plague, rickettsiosis, ehrlichiosis, and Q fever. With a history of rural freshwater exposure, additional infections worth considering include hepatitis A, salmonellosis, toxoplasmosis, and *Naegleria* meningitis. The differential diagnosis may be expanded with other manifestations, such as pulmonary involve-

ment, thrombocytopenia, abdominal pain, or other signs. Laboratory values to consider include complete blood count, urinalysis, and liver function tests including creatinine. Lumbar puncture may be indicated. Leptospirosis has been considered easily treatable with penicillin or doxycycline, though clear evidence-based practice guidelines are still lacking (27,28).

The diagnosis of leptospirosis may be complicated by the difficulty of culturing the spirochetes from the urine of human patients. There is a biohazard concern for laboratory workers, and MAT is becoming progressively less available nationwide. Immunohistochemical assay is a sensitive diagnostic method when tissue sample is available for testing; it can demonstrate the presence of leptospiral antigens with a morphologic context for clinico-pathologic correlation. Furthermore, there is no biosafety issue involved in the diagnostic procedure, and the test can be performed on archival pathologic specimens. More sensitive serologic assays, such as polymerase chain reaction, ought to aid diagnosis as they are becoming more readily available. Though serovar identification might implicate a particular animal reservoir for an outbreak, cross-reaction between serovars may further obscure the definitive source of disease.

Freshwater recreational exposures are the major route of exposure for recent cases acquired in California. Recreation, either domestic or abroad, was the means of exposure by which 59% of California residents acquired leptospirosis in the 20-year period of 1982 to 2001, increasing to 85% in the most recent 5 years. Fresh water may be contaminated with the urine of infected animal reservoirs such as rodents, wild ungulates, and livestock.

Limitations of environmental studies include the lengthy time lapse between exposure to onset, diagnosis, and case investigation, during which time the microbe may be washed away from an aquatic environment. Regions of environmental perturbation including dams or recently expanded recreational areas may be increasing sources of contact with leptospires. Transmission appears to follow warm weather with flooding rains.

Since leptospirosis is a reemerging zoonotic disease, and its presence in animals is widespread, a high index of suspicion for this treatable illness is needed. Because leptospirosis may progress rapidly with severe sequelae, this reemerging infection deserves consideration in febrile patients with a history of freshwater recreation, even in states with a low reported incidence of infection.

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Coronaviridae and SARS-associated Coronavirus Strain HSR1

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During the recent severe acute respiratory syndrome (SARS) outbreak, the etiologic agent was identified as a new coronavirus (CoV). We have isolated a SARS-associated CoV (SARS-CoV) strain by injecting Vero cells with a sputum specimen from an Italian patient affected by a severe pneumonia; the patient traveled from Vietnam to Italy in March 2003. Ultrastructural analysis of infected Vero cells showed the virions within cell vesicles and around the cell membrane. The full-length viral genome sequence was similar to those derived from the Hong-Kong Hotel M isolate. By using both real-time reverse transcription–polymerase chain reaction TaqMan assay and an infectivity plaque assay, we determined that approximately 360 viral genomes were required to generate a PFU. In addition, heparin (100 mg/mL) inhibited infection of Vero cells by 50%. Overall, the molecular and biologic characteristics of the strain HSR1 provide evidence that SARS-CoV forms a fourth genetic coronavirus group with distinct genomic and biologic features.

An outbreak of atypical pneumonia, referred to as severe acute respiratory syndrome (SARS), was identified in the Guangdong Province of People's Republic of China at the end of 2002 and spread to other Asian countries and Canada (1,2) from February through March 2003. Individual cases (all in persons infected in Asia) were diagnosed in Europe during the same period (3). A novel human coronavirus (SARS-associated coronavirus [SARS-CoV]) has been isolated from the oropharyngeal specimens of patients with SARS (4,5). Experimental infection of macaques has confirmed that the SARS-CoV is the cause of SARS (6,7).

Coronaviruses are enveloped, positive-stranded RNA viruses associated with enteric and respiratory diseases in animals and humans; they are currently classified into three antigenic groups: group 1 and 2 include mammalian coronaviruses, and group 3 encompasses avian coronaviruses. Human coronaviruses are associated with common cold-like diseases and are included in both group 1 (CoV-229E) and 2 (CoV-OC43) (8). Sequence analysis of the complete genome of SARS-CoV has shown an RNA molecule of about 29,750 bases in length, with a genome organization similar to that of other coronaviruses (9–11). In spite of this similar organization, the SARS-CoV RNA sequence is only distantly related to that of previously characterized coronaviruses (9). Consequently, whether the SARS-CoV has "jumped" from a nonhuman host reservoir to humans and the molecular basis of such a jump remain unanswered questions (12). Some biologic features of the SARS-CoV described in vivo and in vitro differ from those of other coronaviruses previously identified. Among these features are the peculiar tropism of the virus for Vero cells (a continuous cell line established from monkey kidney epithelial cells), its capacity for growth at 37°C (while other respiratory coronaviruses grow at lower temperatures), and its ability to infect lower respiratory tract tissues (13). These aspects render the molecular and biologic characterization of SARS-CoV important not only for understanding the determinants of its pathogenic potential but also for planning rational strategies of antiviral therapy and vaccination.

We have recently obtained a SARS-CoV isolate from a frozen sputum sample collected from an Italian patient affected by a respiratory disease of unknown cause; onset of illness began during the patient's travel to Vietnam in March 2003. The viral strain has been designated as SARS-CoV HSR1. To gain insight into SARS-CoV biopathology, we analyzed the relevant features of SARS-CoV HSR1 growth in vitro, including the ultrastructural analysis of the consequences of virus replication in Vero cells. We have optimized both a reverse transcription–polymerase chain reaction (RT-PCR) TaqMan assay for quantifying the number of viral genomes and a plaque assay for performing titration of the virus infectivity. In addition, we have completely sequenced the viral genome of the SARS-CoV HSR1 and compared it to other SARS-CoV strains recently isolated in disease-epidemic areas.

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Methods

SARS-CoV Isolation

In March 2003, an Italian man who recently traveled from Vietnam to Italy was affected by a respiratory disease of unknown cause; he was hospitalized in a clinical unit for acute infectious diseases in a public hospital in Milan, Italy. A sputum sample was collected at the peak of illness and stored at –80°C. Isolation of SARS-CoV was performed with Vero cells maintained in Dulbecco's modified Eagle medium (D-MEM, BioWhittaker, Verviers, Belgium) and supplemented with 10% fetal calf serum (FCS, HyClone, Perbio Science Erembodegem-Aalst, Belgium), penicillin/streptomycin (BioWhittaker), and 2.5 μg/mL Fungizone (Invitrogen Ltd, Life Technologies, Paisley, UK) (complete medium). In detail, an aliquot (0.5 mL) of the sputum sample was mixed with 2 x 10^6 Vero cell suspension in 2 mL of complete medium. After incubation for 1 h at 37°C, 5% CO_2, 4 mL of complete medium was added, and the cell suspension was transferred into a 25-cm^2 tissue culture flask (Falcon, Becton Dickinson Labware, Lincoln Park, NJ). Twenty-four hours after inoculation, the cell cultures were examined with an optical microscope for evidence of cytopathic effects (CPE). An aliquot of culture supernatant was collected and stored at –80°C for RT-PCR evaluation. After an additional 24 hours, both the culture supernatant and the cells were collected after 2 cycles of freeze thawing, followed by clarification of the thawed contents by centrifugation at 1,000 x *g* and dispensation of the supernatant into aliquots stored at –80°C (primary viral stock). For generation of the secondary and tertiary viral stocks, adherent Vero cells flasks were seeded in a 25 cm^2 tissue culture and injected with 0.5 mL of stored supernatant filtered with 0.45 μm filters. Three days after infection, the cells and supernatant were subjected to 2 cycles of freeze thawing, and the supernatant was collected after centrifugation followed by filtration as described above (secondary viral stock). Finally, Vero cells seeded in 2 flasks of 75 cm^2 were injected with 1.5 mL of the second passage virus stock in a total volume of 25 mL to generate a third-passage viral stock.

RT-PCR Amplification of Viral Sequences

Direct SARS-CoV RNA amplification was performed starting from the sputum sample treated with an equal volume of phosphate-buffered solution (PBS). RNA was purified from 750 μL of the resulting homogenate by using the Qiagen Viral RNA Mini Kit (Qiagen, Inc., Santa Clarita, CA) (elution volume 50 μL), according to manufacturer's instructions. Viral RNA was extracted from 0.5 mL of culture supernatant with the same kit (elution volume 50 μL). cDNA synthesis and subsequent amplification were performed by using either nested RT-PCR or real-time RT-PCR approach, as described elsewhere, with minor modifications (3). In brief, 5 μL of extracted RNA was reverse-transcribed for 30 min at 42°C by using Mo-MuLV RT, a mixture of random hexamers, and an oligo-(dT) primer. Two microliters of the synthesized cDNA was subsequently amplified by using the following primers pairs: outer primers: BNI-OUTS2, 5′-ATG AAT TAC CAA GTC AAT GGT TAC-3′; BNI-OUTAS, 5′-CAT AAC CAG TCG GTA CAG CTA C-3′; inner primers: BNI-INS, 5′-GAA GCT ATT CGT CAC GTT CG-3′; BNIINAS, 5′-CTG TAG AAA ATC CTA GCT GGA G-3′. A sequence of the SARS-CoV RNA encompassing the target region was used as positive control, and a sputum sample from a SARS-negative healthy donor and a feline coronavirus RNA extract were used as negative controls. The amplified PCR product was separated by agarose gel electrophoresis visualized by ethidium bromide staining and sequenced directly on an ABI PRISM 3100 Genetic Analyzer with ABI PRISM BigDye Terminator v3.0 sequencing kit (Applied Biosystems, Foster City, CA). SARS-CoV was then identified by searching for homologies between the amplified fragment and previously deposited sequences using BLAST. Finally, the quantitation of the viral RNA was performed in a TaqMan assay after generation of cDNA. The primer pair and probe BNITMSARS1, 5′-TTATCACC-CGCGAAGAAGCT-3′; BNITMSARAS2, 5′-CTC-TAGTTGCATGACAGCCCTC-3′, BNI-TMSARP 6-carboxifluorescein-TCG TGC GTG GAT TGG CTT TGA TGT-6 carboxy-tetramethylrhodamin (3) were added to the universal PCR master mix (Applied Biosystems) at 200 and 120 nM, respectively, in a final volume of 25 μL. The standard was obtained by cloning the 77-bp fragment into the pCR2.1 plasmid by using the TA cloning kit (InVitrogen Corp., San Diego, CA). A linear distribution ($r = 0.99$) was obtained between 10^1 to 10^8 copies.

SARS-CoV Plaque Infectivity Assay

To have the most effective quantitative assay of virus infectivity and to compare the infectious titer with quantitative molecular assays, we optimized a plaque assay (determining the PFU/mL). Confluent Vero cells in 6 well plates (Falcon) were incubated in duplicate with 1 mL of PBS containing 100 μL of SARS-CoV HSR1 viral stock in 10-fold serial dilutions from –10e2 (1/100) to –10e8 (1/100,000,000). After 1 hour of incubation, the viral inoculum was removed and 1 mL of 1% carboxymethylcellulose (Sigma Chemical Corp., St. Louis, MO) overlay with DMEM supplemented with 1% fetal calf serum was added to each well. After 6 days of incubation, the cells were stained with 1% crystal violet (Sigma) in 70% methanol. The plaques were counted after being examined with a stereoscopic microscope (SMZ-1500, Nikon). The virus titer was calculated in PFU per milliliter.

Ultrastructural Analysis

Adherent Vero cells were infected with 5 x 10^4 PFU/mL of SARS-CoV HSR1 at the third passage in a T-75 flask. Twenty-four hours after infection, Vero cells were detached from the tissue culture flask with a cell scraper, and the cell suspension was centrifuged at 173 x *g* for 4 min. The pellet was resuspended in PBS without Ca^{++} and Mg^{++}, and the suspension was spun at 173 x *g* for an additional 4 min. The cell pellet was resuspended and fixed in 4% formaldehyde and 2.5% glutaraldehyde in cacodylate buffer and incubated for 5 min at room temperature. The sample was then centrifuged at 13,414 x *g* for 5 min; the pellet was fixed with 2% OsO_4 in 2.5% glutaraldehyde in cacodylate buffer for 60 min. The pellet was dehydrated in graded ethanol, washed in propylene oxide and infiltrated for 12 hours in a 1:1 mixture of propylene oxide and epoxidic resin (Epon). Cells were then embedded in Epon and polymerized for 24 hours at 60°C. Slides were cut with ultramicrotome (Ultracut Uct, Leica, Deerfield, IL), stained with uranyl acetate and lead citrate, and metaled. The ultrathin sections of infected Vero cells were observed through transmission electron microscopy (Hitachi H7000).

SARS-CoV HSR1 Genome RNA and Phylogenetic Reconstruction

Sequencing of the complete SARS-CoV HSR1 genome was performed by using 68 partially overlapping primers encompassing the whole viral genome. A 5′ rapid amplification of PCR ends (RACE) technique was performed to capture the 5′-untranslated region of the genome (10). Each 750-bp fragment was gel-isolated by means of a QIAQuick gel Extraction kit (Qiagen) and directly sequenced from both directions inward and outward. SeqScape version 2.0 (Applied Biosystems) software was used for base calling, editing, and assembly of the fragments. Manual check of the differences between the electropherograms and the reference sequence (Urbani isolate) was performed and eventually led to reamplification or resequencing of some fragments. The complete sequence of SARS-CoV HSR1 strain was aligned with all the previously sequenced full-length genomes available from the GenBank database by using ClustalW and editing with BioEdit version 5.0.9 for manual corrections. Available full-length sequences of SARS-CoV strains accessed from GenBank and used for phylogenetic analysis and genotyping were as follows: Taiwan TC2 (AY338175), Taiwan TC1 (AY338174), TWC (AY321118), Sin2774 (AY283798), Sin2748 (AY283797), Sin2679 (AY283796), Sin2677 (AY283795), Sin2500 (AY283794), Frankfurt 1 (AY291315), BJ04 (AY279354), BJ03 (AY278490), BJ02 (AY278487), GZ01 (AY278489), CUHK-W1 (AY278554), ZJ01 (AY297028), TOR2 (AY274119), TW1 (AY291451), CUHKSu10 (AY282752), BJ01 (AY278488), Urbani (AY278741), and HKU-39849 (AY278491). Sequences were trimmed to equivalent length and phylogenetic relationships were estimated with PAUP* (maximum-parsimony and maximum-likelihood methods by using the p-distance model) and MEGA version 2.1. Trees were edited by using Treeview.

Results

Both nested RT-PCR and the real-time RT-PCR assays performed on the sputum sample from a person with SARS tested positive for SARS-CoV RNA. The viral load in the sample was estimated to be 5.6 x 10^4 SARS-CoV RNA copies/mL. Twenty-four hours after injection of Vero cells with the sputum sample, a strong CPE was observed, as indicated in Figure 1B. The CPE was diffused with cell rounding with refractive appearance, and the cell monolayer was destroyed as compared to control uninfected Vero cells (Figure 1A). The viral load in the culture supernatant 24 hours after injection with the clinical sample was 1.3 x 10^5 copies/mL as determined by quantitative real-time RT-PCR. Serial passage of the virus on Vero cells to obtain a tertiary viral stock yielded 9.1 x 10^8 copies/mL. A plaque assay was optimized to determine the in vitro infectivity of SARS-CoV. The tertiary viral stock tested 2.5 x 10^6 PFU/mL in the plaque assay, suggesting that about 360 genomes were required to generate a single plaque in tissue cultures, at least under the conditions described here. We also used the plaque assay for testing the potential inhibitory effect on virus infectivity of a single concentration of heparin, the prototypic compound of a class of inhibitors of virus entry for enveloped viruses including HIV type 1 (HIV-1) (14) and herpes simplex virus 1 and 2 (HSV-1 and 2) (15). Heparin indeed reduced the formation of plaques by 50% when added 30 min before infection of Vero cells with 100 PFU/mL of the SARS-CoV (Figure 2).

Thin-section electron microscopy showed the typical features of intracellular CoV particles. Cells were engulfed with viral particles localized in cytoplasmic vesicles (Figure 3A). This feature is typical of all *Coronaviridae* viruses that bud intracellularly at membranes of the intermediate compartment between the endoplasmic reticulum and the Golgi complex, whereas newly assembled virions reach the cell surface by vesicular transport (16). After the extracellular release, virus particles were found in large clusters adjacent to the plasma membrane, as evidenced in Figure 3B. Overall, the ultrastructural analysis documented that SARS-CoV cultivated in Vero cells behaved like a typical coronavirus, characterized by intracellular budding (Figure 3, panels C and D).

The sequence of SARS-CoV strain HSR1 genomic RNA was 29,751 bases in length, with a polyA tail. We identified the major open reading frames (ORFs), coding

for the 4 major structural proteins, namely: spike (S), envelope (E) matrix (M), nucleocapsid (N) gene products, and at least other 10 proteins, including a few of unknown function. The complete sequence of SARS-CoV strain HSR1 has been deposited in the GenBank database (GenBank accession no. AY323977). This sequence was aligned with those of the other 21 SARS-CoV isolates to facilitate phylogenetic analysis. Overall, the mean difference in nucleotide composition between all the isolates was 18 ± 1 nt variations within the whole genomes, thus confirming the genetic conservation observed previously (9). On the whole, 149 sites were variable among the 22 aligned isolates, and 24 loci in the viral genome varied in more than one isolate, including in the SARS-CoV HSR1 genome (recurrent mutations). Eleven of 24 recurrent mutations were silent, whereas 3 of 13 mutations generating amino acid substitutions were observed within the N-terminal domain of the spike glycoprotein gene (positions 21,722, 22,223, and 22,423, determining a G to D, an I to T, and a G to R amino acid change, respectively). Six mutations were detected in the replicase gene (positions 8,572, 9,404, 9,479, 9,854, 17,564, and 19,084, determining a V to L, a V to A, a V to A, an A to V, a D to E, and a T to I amino acid change, respectively), and 1 mutation was found in the matrix protein (position 26,600, determining an A to V amino acid change). The phylogenetic analysis was also performed by using the maximum parsimony method, considering only sequence variants that recurred in more than one strain in order to reduce the mutational noise caused by PCR or sequencing mistakes. Figure 4 shows the phylogenetic tree obtained with the maximum likelihood method. The maximum parsimony trees produced the same structure with minor changes in the subtrees' branch patterns (data not shown). In these analyses, SARS-CoV HSR1 appears to be strongly related to strains isolated from patients who had traveled from Hong Kong to different geographic areas (Singapore, Canada, Vietnam) and spread the infection to their home countries in a few cases.

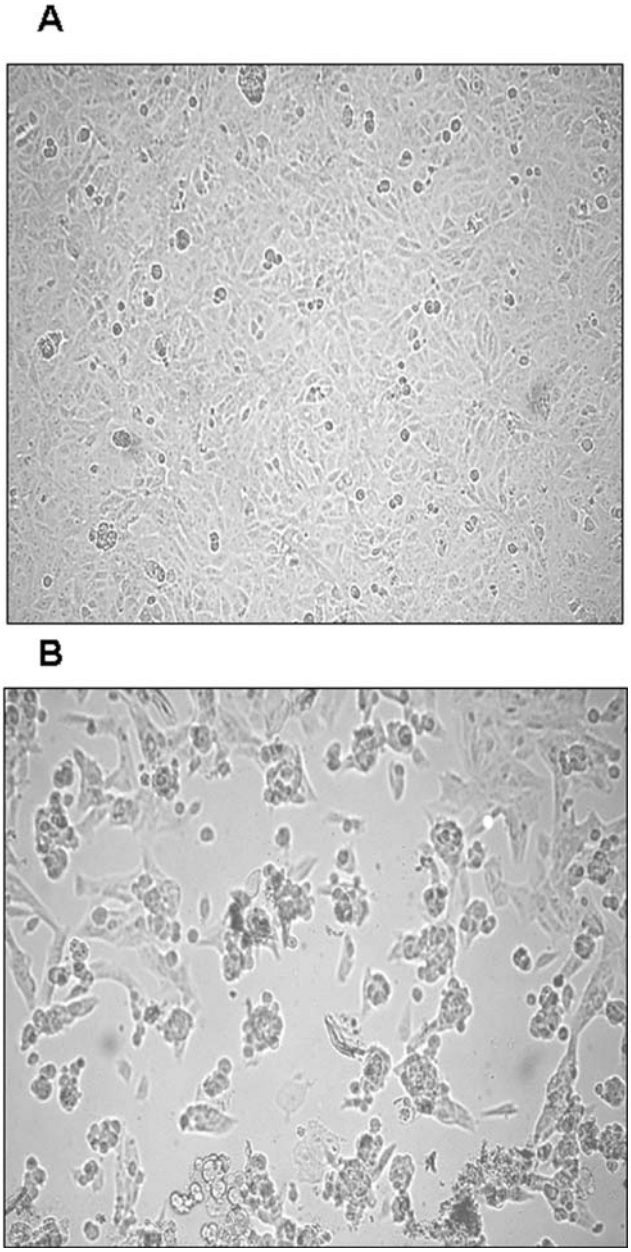

Figure 1. Cytopathic effect (CPE) of primary severe acute respiratory syndrome–associated coronavirus strain HSR1 isolate. A, uninfected Vero cells form a continuous monolayer of spindle-shaped cells. B, a strong CPE was observed after 24 hours of incubation of Vero cells with the patient sputum sample (primary isolate).

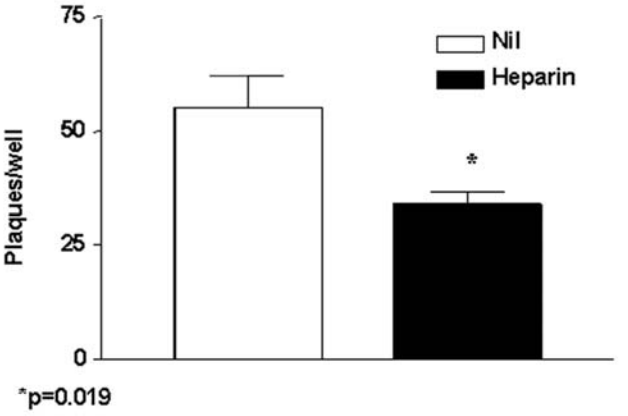

Figure 2. Effect of the sulfated polysaccharide heparin (100 μg/mL) added 30 minutes before injecting Vero cells with 100 PFU/mL of severe acute respiratory syndrome–associated coronavirus strain HSR1. The results are expressed as number of plaques/well and represent the mean ± SEM of two independent experiments each carried out in duplicate cultures. The p value was calculated by the Mann-Whitney U test.

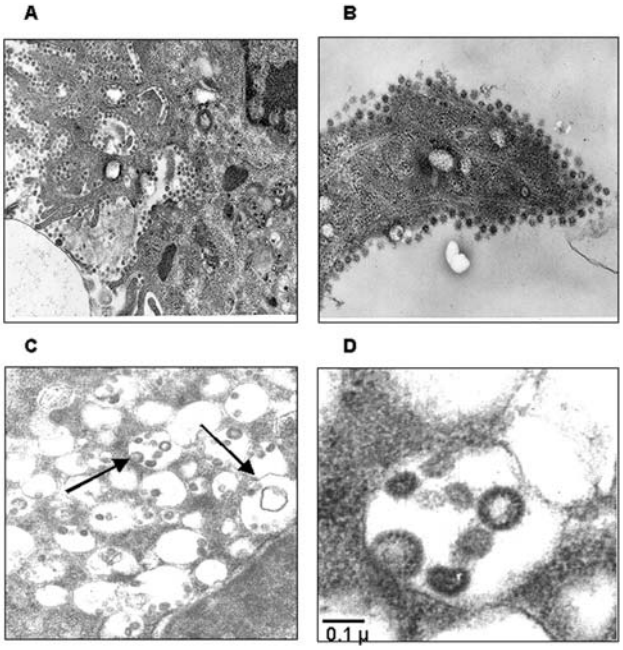

Figure 3. Ultrastructural analysis of Vero cells infected with severe acute respiratory syndrome–associated coronavirus (SARS-CoV) strain HSR1. A, intracellular budding of SARS-CoV in large vesicles containing CoV virions (magnification x30,000); B, clusters of extracellular virions adjacent to the plasma membrane (magnification x50,000); C and D, intracellular budding of SARS-CoV virions (magnification x50,000).

Discussion

The isolation of a novel CoV in persons with SARS and proof of its etiologic role in this disease underscore the importance of identifying the pathogenic determinants of SARS-CoV. This information may be central to plan specific therapeutic and preventive strategies against SARS. Major areas of research include the following: 1) analyzing growth characteristics of SARS-CoV in vitro; 2) evaluating virus-host relationships at the molecular level; 3) understanding both SARS-CoV genome organization and the role of nonstructural proteins of unknown function; 4) studying the evolutionary relationships of different coronaviruses of animal and human origin; and 5) identifying targets for anti-CoV chemotherapy and vaccination. In our study, we characterized a novel isolate of SARS-CoV (designated HSR1 strain). The analysis of the complete sequence of SARS-CoV HSR1 genome showed the same organization in ORFs previously described in this novel human virus (10) and confirmed the relative genetic stability among the different isolates (9). However, for other SARS-CoVs isolates, amino acid changes were observed in this HSR1 strain at the level of the N-terminal domain of the spike glycoprotein, the viral replicase, and the matrix protein. Despite this relative stability, phylogenetic analysis of the HSR1 isolate (Figure 4) has shown that the virus is more related to the strains isolated in patients who had traveled from Hong Kong to different geographic areas, such as Singapore, Canada, and Vietnam, than to the Beijing isolates. In addition, we have observed that other biomolecular features shared by most *Coronaviridae* coexist in SARS-CoV HSR1 with particular characteristics that seem to be unique of the novel virus. In particular, SARS-CoV is able to replicate in Vero cells with a rapid production of high virus titers and fast CPE. By using quantitative molecular and biologic assays, we could estimate that, under our experimental conditions, 360 genomes are approximately required to form a plaque of infectivity in vitro. SARS-CoV seems unlike most of the other respiratory coronaviruses infecting humans, which fail to grow efficiently in tissue cultures and are easily detectable by using PCR amplification methods only (17). Infection in Vero cells showed most of the usual characteristics of the CoV replication (16), including intracellular budding of virions, as demonstrated by electron microscopy.

To investigate whether SARS-CoV could be inhibited by polyanions, we tested the effects of the prototypic sulfated polysaccharide heparin (Figure 2) on in vitro infection. Incubation of Vero cells with heparin (100 µg/mL) 30 min before SARS-CoV injection curtailed infection by 50%. Polyanions have demonstrated antiviral activity (18) against enveloped viruses such as HIV and HSV through specific interaction with cells and after inhibition of virus attachment and entry (14). Heparin, a prototypic polyanion, inhibits attachment and entry of virus particles into cell by impeding the interaction of the V3 region of gp120 with HIV specific chemokine coreceptor (19). In the case

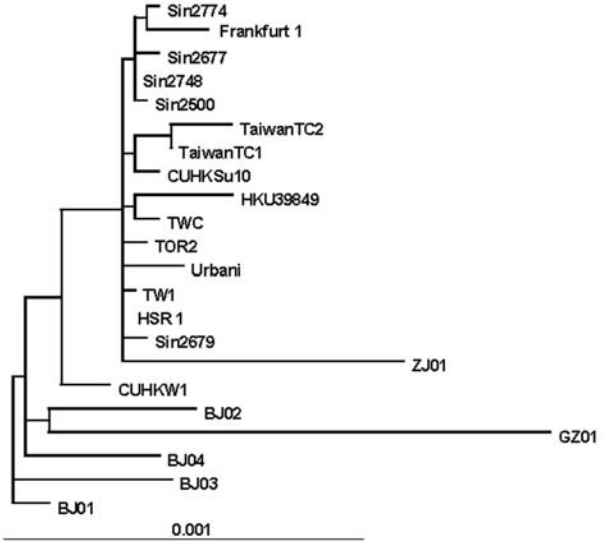

Figure 4. Phylogenetic tree obtained by applying PAUP* (maximum-likelihood methods using the p-distance model) applied to complete genome sequences of the severe acute respiratory syndrome–associated coronavirus (SARS-CoV) HSR1 strain and the 21 other SARS-CoV isolates.

of HSV, heparin inhibits the interaction of some HSV envelope glycoprotein to the heparan sulfate that mediates viral attachment to the target cell surface (20,21). The partial inhibition of SARS-CoV HSR1 by heparin suggests that the envelope proteins coating the SARS-CoV virions might be endowed with positively charged amino acids that could interact with negatively charged sulfate groups present on heparan sulfate proteoglycans expressed on the surface of target cells. Vero cells, the target of both HSV and dengue 4 (22) replication, replicate SARS-CoV more efficiently than several other cell lines.

In conclusion, our study characterized a novel SARS-CoV isolate, which was to date the second isolate obtained in Europe. The sequence analysis indicates that SARS-CoV HSR1 clusters together with the Hong Kong, Singapore, and Canada isolates. The coexistence of general coronavirus features with important biologic and molecular specificity of SARS-CoV, together with the evidence that SARS-CoV does not appear to be linked to any of the other three genetic groups of known coronaviruses, strongly suggests it represents a new, fourth genetic coronavirus group, with distinct genomic and biologic features. In light of this evidence, efforts to identify new types of animal coronaviruses as well as to better understand the peculiarities of SARS-CoV are important in addressing its possible animal origin and in understanding the biology of *Coronaviridae* and their evolutionary features.

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Dr. Vicenzi works at the San Raffaele Scientific Institute, Milan, as senior investigator in the AIDS Immunopathogenesis Unit. Her research interests are primarily HIV/AIDS, specifically, the role of the *nef* gene in HIV disease and the search for inhibitors of HIV entry.

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Laboratory Analysis of Tularemia in Wild-Trapped, Commercially Traded Prairie Dogs, Texas, 2002

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Oropharyngeal tularemia was identified as the cause of a die-off in captured wild prairie dogs at a commercial exotic animal facility in Texas. From this point source, *Francisella tularensis*–infected prairie dogs were traced to animals distributed to the Czech Republic and to a Texas pet shop. *F. tularensis* culture isolates were recovered (or made) from 63 prairie dogs, including one each from the secondary distribution sites. Molecular and biochemical subtyping indicated that all isolates were *F. tularensis* subsp. *holarctica* (Type B). Microagglutination assays detected antibodies against *F. tularensis*, with titers as great as 1:4,096 in some live animals. All seropositive animals remained culture positive, suggesting that prairie dogs may act as chronic carriers of *F. tularensis*. These findings demonstrate the need for additional studies of tularemia in prairie dogs, given the seriousness of the resulting disease, the fact that prairie dogs are sold commercially as pets, and the risk for pet-to-human transmission.

Francisella tularensis is the causative agent of the zoonotic disease tularemia (1,2). As few as 10 organisms are sufficient to cause severe disease and death, making *F. tularensis* one of the most infectious bacterial pathogens known. Thus, *F. tularensis* is considered to be a biological threat agent that poses a substantial risk to public health (3).

Infections with *F. tularensis* are widely distributed and occur in >100 wildlife species in the Northern Hemisphere (4,5). Two subspecies of *F. tularensis* are most commonly associated with human and animal disease: *tularensis* (Type A) and *holarctica* (Type B) (6,7). Type A is found almost exclusively in North America and is associated with a severe form of disease in humans and rabbits (*Lepus* spp.). It is commonly differentiated from Type B by its ability to produce acid from glycerol. Type B is found throughout the Northern Hemisphere (holarctic region); it does not produce acid from glycerol and rarely causes death in humans. Type B is most frequently isolated from rodent species, including muskrats (*Ondatra zibethicus*), mice (*Mus musculus*), beaver (*Castor canadensis*), voles (*Microtus* spp.), and water voles (*Arvicola terrestris*).

Infections with *F. tularensis* also occur in the black-footed prairie dog (*Cynomys ludovicianus*) (8). This finding has particular public health significance since wild-caught prairie dogs are sold as pets both domestically and internationally. Wild prairie dogs are found throughout the Great Plains of North America from southern Canada to just inside Mexico. Every year, pups are collected in the United States during April through July and are distributed to pet stores throughout the country as well as being exported internationally.

The first literature report of tularemia in captive prairie dogs described *F. tularensis* infection in three wild-caught animals in 1986 (8). Subsequently, *F. tularensis* infection caused by Type B was confirmed by the Centers for Disease Control and Prevention (CDC), Fort Collins, Colorado, in wild-caught prairie dogs, originating from a single animal exporter and shipped to research institutions in Boston and Houston from 1996 to 1997. In the summer of 2000, CDC again confirmed Type B infection in a wild-caught prairie dog. In this case, a family traveling from Ohio purchased two prairie dogs from a dealer in Kansas; one animal died during transport, while the second animal displayed disease and died after they arrived home.

In August of 2002, an outbreak of tularemia was identified as the cause of a die-off among wild-caught, commercially traded prairie dogs at an exotic animal facility in

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Texas. We describe laboratory findings from this investigation. The epidemiologic findings of the investigation are reported separately (9). During this outbreak, many animals died of infection with *F. tularensis*. However, a small number of surviving animals developed antibodies against *F. tularensis*, suggesting that prairie dogs can survive an infection of tularemia. All seropositive animals were also found to harbor live infectious bacteria, suggesting that prairie dogs may be persistently infected. These findings have important public health implications in light of commercial prairie dog trade practices.

Materials and Methods

Outbreak Groupings

On August 2, 2002, a total of 163 prairie dogs were found at the exotic animal facility in Texas. These animals were classified into four groups: group A (bin 1, dead), group B (bin 1, live), group C (escapees), and group D (bin 2 and cages, healthy). Group A animals (n = 46) were collected during the last week of July through August 2, 2002. All group A animals had been housed in an uncovered metal tub (bin 1). The live animals remaining in bin 1 were classified as group B (n = 23), with most of the animals being emaciated, dehydrated, and lethargic. Group C (n = 36) comprised escaped prairie dogs that were running free throughout the facility. Group D prairie dogs (n = 58) were physically separated from both group B and C animals, and all group D animals were large, well-nourished, energetic, and noisy. Group D animals were housed in an uncovered metal tub (bin 2) and in several wire cages.

Animals from the Texas facility that had been sent to other locations made up two additional groups. Group E animals comprised seven prairie dogs that originated from the Texas facility, were distributed to pet shops in Texas, and recalled once *F. tularensis* was identified as the cause of the outbreak. Group F comprised 100 prairie dogs shipped from the Texas facility to the Czech Republic.

Culture Recovery of *F. tularensis*

All prairie dogs at the Texas facility (n = 163) were necropsied on site, and tissues were surgically removed. Appropriate biosafety measures were adhered to, including the use of closed front gowns, N95 masks, glasses, and gloves. Spleen and liver samples were spread onto cysteine heart agar supplemented with 9% sheep blood (CHAB). Plates were sealed with parafilm and transported in ice coolers (~15°C–20°C) until arrival at the CDC laboratory, Fort Collins, Colorado (~72 hours). Culture plates were then transferred to a biosafety level (BSL) 3 incubator at 37°C for 5 days and checked daily for *F. tularensis* growth. Some tissues were also spread onto CHAB medium containing antibiotics (10), incubated at 37°C for 7 days, and checked daily for *F. tularensis* growth. A culture isolate from prairie dogs shipped to the Czech Republic was grown at the State Veterinary Administration, Prague, Czech Republic, and submitted to our laboratory.

Spleen and liver tissues were injected into pathogen-free Swiss-Webster outbred mice for culture recovery of *F. tularensis* (IACUC Protocol 00-06-018-MUS). Tissues (~1g) from individual prairie dogs were ground with mortar and pestle, resuspended in 2 mL of saline and 0.5 mL of the tissue suspension was injected subcutaneously per mouse. All injections were performed in a BSL2 animal facility, and appropriate biosafety measures were followed, including the use of closed front gowns, N95 masks, glasses, and gloves. Animals were euthanized when signs and symptoms of tularemia were evident. After euthanasia was performed, 0.5 to 1.0 mL of whole blood was removed by cardiac puncture with a 1.0 mL tuberculin syringe. Liver and spleen tissues were surgically removed and spread onto CHAB with sterile wooden sticks. All healthy injected mice were euthanized 21 days after injection, and serum was tested for anti-*F. tularensis* antibody.

Direct Fluorescent Assay (DFA)

Slide touch preparations of tissues were prepared and heat-fixed immediately after necropsy at the Texas animal facility. On arrival at the laboratory, all slides were incubated with FITC-labeled rabbit anti-*F. tularensis* subsp. *tularensis* (SchuS4 strain) antibodies (CDC) for 30 min at room temperature. Slides were washed twice in phosphate-buffered saline, followed by a final rinse with dH_2O and viewed with a fluorescent microscope using the 40X objective and a 490 nm filter. Direct fluorescent activity was scored independently by two technicians experienced with *F. tularensis* DFA.

Serologic Findings

For all group B, C, and D animals, blood samples were collected from euthanized animals by cardiac puncture. Blood was collected into Microtainer brand serum separator tubes (Becton Dickinson, Franklin Lakes, NJ) and maintained at 4°C until arrival at the laboratory (~72 h). Serum was separated, heat-inactivated for 30 min at 56°C, and tested for *F. tularensis* specific antibodies by using a standard microagglutination assay (11). Briefly, serial dilutions of serum were incubated overnight with safranin-stained, formalin-killed *F. tularensis* subsp. *tularensis* (SchuS4 strain) cells at room temperature, and a titer was assigned reflecting the last well demonstrating full agglutination. Samples with a titer of 1:128 or greater were reported as positive.

Confirming *F. tularensis*

Prairie dogs were confirmed positive on recovery of an isolate with characteristic growth on CHAB and positive testing of the isolate by DFA or IS*Ftu2* polymerase chain reaction (PCR). Animals were considered presumptive positive if tissues tested positive by DFA or PCR, but no isolate was obtained. Prairie dogs were considered negative if all three diagnostic tests (culture, DFA, serologic testing) failed to detect any evidence of *F. tularensis* infection. For negative samples, recovery of culture included passage of the spleen and liver tissues through mice.

F. tularensis Subtyping

For molecular subtyping, DNA was prepared after injection of a 1 μL loop of culture into 200 μL TE buffer. Cells were lysed by boiling at 95°C for 10 min. A differential PCR, based on the presence or absence of the IS*Ftu2* element (GenBank accession no. AY062040), was performed by using 1 μL of the lysed bacterial supernatant and the primers TuF1705 (5′-GATAGATACACGCCTTGCTCACA-3′) and TuBR431(5′-ACCCAGCCAATGCCTAAATA-3′) (Y. Zhou, unpub. data). The amplification program included a denaturation cycle at 95°C for 2 min, followed by 35 amplification cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final elongation cycle of 72°C for 5 min. PCR products were analyzed by agarose gel electrophoresis, followed by staining with EtBr and visualization with a Bio-Rad Gel Doc UV system (Bio-Rad Laboratories, Hercules, CA). For biochemical subtyping, the 96-well automated MicroLog MicroStation System with GN2 Microplates (Biolog Inc, Hayward, CA) was used. Microplates were set up and analyzed per the manufacturer's instructions.

Statistical Analysis

McNemar's test was used for statistical analysis. Sensitivities of different diagnostic tests were evaluated for their ability to detect *F. tularensis* in a given population of animals (either live or dead animals).

Results

Laboratory Findings

F. tularensis–infected prairie dogs from the Texas animal facility were traced to Texas pet shops and animals shipped to the Czech Republic. From these three sources, 177 prairie dogs (1 animal whose illness initiated the investigation [12], 163 animals that remained on site at the Texas facility, 7 animals recalled from Texas pet shops, and 6 animals shipped to the Czech Republic) were tested. Of these animals, 63 were confirmed positive, 13 were identified as presumptive positives, and 101 were confirmed negative for *F. tularensis* infection (Table 1).

F. tularensis Isolates from Infected Prairie Dogs

Because prairie dogs were sold commercially as pets and the risk for pet-to-human transmission was unknown, determining which groups (A–D) of animals were potentially infectious was important. Subtyping the *F. tularensis* isolates was also important, since this outbreak carried the threat of international dissemination. Therefore, our laboratory efforts focused on recovery of viable organisms. In total, 63 isolates were recovered (Table 1): 61 from prairie dogs at the Texas facility (groups A–C), 1 isolate from prairie dogs recalled from Texas pet shops (group E), and 1 isolate from prairie dogs distributed to the Czech Republic (group F).

Biochemical and Molecular Typing of *F. tularensis* Isolates

For subtyping of the 63 *F. tularensis* isolates (63 isolates described here, including 1 isolate that initiated the investigation, TX021935 [12]), a combination of biochemical and molecular typing was used. Biochemical characterization was performed on 15 isolates representative of all five groups of *F. tularensis*–positive animals (groups A, B, C, E, F). All 15 *F. tularensis* isolates were unable to use glycerol as a carbon source and thus were classified as Type B (data not shown). In addition, six representative isolates were tested for antimicrobial susceptibilities and

Table 1. Laboratory results for outbreak of tularemia in wild-trapped, commercially sold prairie dogs

Group	Prairie dogs	No. of animals	Presumptive-positive samples[a]	Confirmed-positive samples[b]	Confirmed-negative samples[c]
A	Exotic animal facility, Texas, bin 1, dead animals	47[d]	7	40	0
B	Exotic animal facility, Texas, bin 1, live animals	23	0	20	3
C	Exotic animal facility, Texas, escapees	36	0	1	35
D	Exotic animal facility, Texas bin 2 and cages, healthy	58	0	0	58
E	Pet shop recalls, originating from exotic animal facility, Texas	7	1	1	5
F	Czech Republic, originating from exotic animal facility, Texas	100	5	1	Not determined

[a]Prairie dogs were confirmed positive on recovery of an isolate with characteristic growth on cysteine heart agar with 9% sheep blood and positive testing of the isolate by direct fluorescent assay (DFA) or IS*Ftu2* polymerase chain reaction (PCR).
[b]Prairie dogs were considered presumptive positive if primary tissues tested positive by DFA or PCR but no isolate was obtained.
[c]Prairie dogs were confirmed negative if all three diagnostic tests (culture, DFA, serologic testing) failed to detect any evidence of *Francisella tularensis* infection.
[d]46 animals that remained on site August 2, 2002, plus 1 animal that initiated the outbreak investigation (TX021935).

demonstrated MICs consistent with those published previously for Type B (data not shown, 13).

To distinguish molecularly between Type A and Type B, a differential PCR based on the presence or the absence of the IS*Ftu2* element was performed (Y. Zhou, unpub. data). For Type A, a PCR product of 390 bp was amplified, whereas for Type B, a product of 1,249 bp was amplified. When IS*Ftu2* PCR subtyping was performed on all 63 isolates, all were shown to be Type B, including the single isolate received from the Czech Republic. Representative IS*Ftu2* PCR subtyping for the five groups (A, B, C, E, and F) of *F. tularensis*–positive animals is shown in Figure 1. Additional analysis with IS*Ftu2* restriction fragment length polymorphisms southern blotting demonstrated that the *F. tularensis* isolates were molecularly indistinguishable (data not shown).

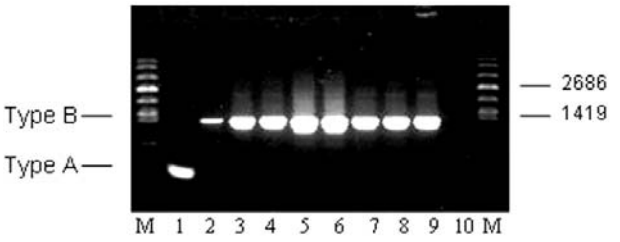

Figure 1. Molecular subtyping of representative *Francisella tularensis* isolates from Group A, B, C, E, and F prairie dogs. The expected size PCR fragments for *F. tularensis* subsp. *tularensis* (Type A) and *holarctica* (Type B) are shown in lanes 1 and 2, respectively. Subtyping results for the five groups (A, B, C, E, F) are shown in lanes 3–9. Lane 3: TX021935 (A); lane 4: TX022151 (A); lane 5: TX022537 (B); lane 6: TX022592 (B); lane 7: TX022799 (C); lane 8: TX022107 (E); lane 9: CZ024233 (F). Lane 10: no DNA template control. Lane M: molecular weight markers.

Texas Facility Investigation (Groups A–D)

The animals remaining at the Texas facility (groups A–D) provided insight into how tularemia was transmitted among the prairie dogs. When necropsies were performed on animals in groups A–D, cannibalization, as indicated by partially eaten prairie dog carcasses, was noted among group A animals. In addition, all group A and most of group B animals displayed swollen submandibular lymph nodes, suggesting that all animals ingested the bacteria.

Because all prairie dogs from the Texas facility (groups A–D) were tested and classified as confirmed positive, presumptive positive, or negative (Table 1), diagnostic test sensitivities could be determined. From the 68 prairie dogs at the Texas facility that tested positive for *F. tularensis* by one or more diagnostic methods (culture, DFA, serologic testing), 61 isolates were recovered, yielding an overall culture recovery rate of 89.7%.

Detecting *F. tularensis* in Live, Infected Animals

For determining *F. tularensis* infection in live, infected animals, the sensitivities of culture versus DFA and serologic testing were compared (Table 2). Testing all 59 animals in groups B and C, confirmed 21 animals as *F. tularensis*–positive and 38 animals as *F. tularensis*–negative (Table 1). For the 20 animals confirmed positive by analysis of spleen and liver tissues, culture detected *F. tularensis* in 100% of cases. In contrast, both DFA and serologic testing detected *F. tularensis* in 10 of 20 animals, yielding a sensitivity of only 50% (Table 2). These differences were significant ($p \leq 0.05$) and demonstrate that culture of spleen and liver tissues is more sensitive than DFA or serologic testing for detecting *F. tularensis* in live, infected prairie dogs.

Since the outbreak was consistent with oropharyngeal tularemia, submandibular lymph nodes of group B animals were also analyzed. When lymph nodes were cultured, an additional case was confirmed by isolation of *F. tularensis* from animal B17 (Table 3). The bacterium was not cultured from the spleen and liver of this prairie dog even on passage of tissues through Swiss-Webster mice. This finding suggested that prairie dog B17 had recently ingested *F. tularensis* and that the infection was localized to the submandibular lymph nodes.

To determine if lymph node tissues were a better tissue source than either spleen or liver tissues for detection of *F. tularensis*, DFA was used for direct comparison of tissues from culture-positive group B animals (Table 3). When spleen and liver tissues were analyzed, the sensitivity of DFA was 50%, whereas for analysis of submandibular lymph node tissues the sensitivity of DFA was 89.5% (Table 2). This difference in sensitivities was significant ($p \leq 0.05$) and demonstrates that for cases of oropharyngeal tularemia, submandibular lymph node tissues are the most appropriate source for detecting infection by DFA.

Detecting *F. tularensis* in Fatal Cases of Tularemia

For fatal cases of tularemia, the sensitivity of culture and DFA was also compared (Table 2). Of the 47 animals in group A, 40 were confirmed positive, and 7 were presumptive positive for *F. tularensis* (Table 1). Direct fluorescence analysis of spleen and liver tissues identified all 47 animals as *F. tularensis* positive, yielding a sensitivity of 100%. In contrast, 40 *F. tularensis* isolates were obtained, yielding a sensitivity of 85.1%. These results were significant and demonstrated that DFA was more sensitive than culture for detection of *F. tularensis* in carcasses ($p \leq 0.05$).

Seropositivity and Decreased *F. tularensis* Levels in Live, Infected Animals

To test for evidence of seroconversion in live, infected animals, serum samples from group B prairie dogs were

Table 2. Comparison of diagnostic sensitivities of culture and direct fluorescent assay (DFA) for detection of *Francisella tularensis* in live versus dead prairie dogs (groups A–C)

	No. (%) of samples positive for:			
Prairie dogs[a]	Culture (spleen/liver)	Direct fluorescence (spleen/liver)	Direct fluorescence (lymph node)	Serologic testing
Groups B, C; live, infected animals (n = 20)	20 (100)	10 (50)	17 (89.5)[b]	10 (50)
Group A, dead animals (n = 47)	40 (85.1)	47 (100)	Not tested	Not tested

[a]All 67 prairie dogs tested positive for *F. tularensis* by at least one diagnostic test (culture, DFA, or serologic testing).
[b]19 *F. tularensis*–positive animals were tested.

checked for anti-*F. tularensis* antibodies. Ten animals showed evidence of seroconversion, displaying titers against *F. tularensis* as great as 1:4,096 (Table 3). To our knowledge, this evidence is the first that prairie dogs can develop specific antibodies on infection with *F. tularensis*. In addition, *F. tularensis* was successfully recovered from the spleen of all 10 seropositive animals, suggesting that prairie dogs may become persistently infected.

Comparison of DFA results for seropositive and seronegative prairie dogs indicated that the levels of *F. tularensis* in liver and spleen were greatly decreased in seropositive prairie dogs. In 7 of 10 seropositive prairie dogs, *F. tularensis* was not detectable by DFA analysis of spleen and liver tissues (Table 3, Figure 2, panel b). Conversely, 7 of 10 seronegative animals were positive by DFA analysis of spleen and liver tissues (Table 3, Figure 2, panel a). These findings demonstrate that seropositivity in prairie dogs leads to decreased levels of *F. tularensis* and may suggest that seropositive prairie dogs can survive an acute infection of oropharyngeal tularemia.

Discussion

In our study, we documented the laboratory results from an outbreak of oropharyngeal tularemia among wild-caught, commercially distributed prairie dogs. *F. tularensis*–infected prairie dogs from the Texas animal facility were traced to Texas pet shops and to the Czech Republic. Our findings indicate that the primary mechanism of transmission was ingestion of *F. tularensis*, as all infected prairie dogs displayed enlarged submandibular lymph nodes, a hallmark of oropharyngeal tularemia. In addition, all prairie dogs (group D) physically separated from sick animals were negative for *F. tularensis* infection, demonstrating that the outbreak of tularemia at the Texas facility required contact with infected animals. While other modes of bacterial ingestion cannot be ruled out, this outbreak most likely resulted from cannibalism of dead animals. Cannibalism, as evidenced by partially eaten carcasses, was observed at the Texas facility as well as in the shipment of animals to the Czech Republic. In nature, cannibalism occurs in rodents and has been previously documented as the cause for spread of tularemia (14,15).

Table 3. Diagnostic test results for culture–positive group B prairie dogs[a]

Prairie dog	DFA (spleen/liver)	DFA (submandibular lymph node)	Serologic testing (microagglutination assay)	
B1	+	+	1:512	Pos
B2	+	+	1:32	Neg
B3	+	+	1:8	Neg
B4	–	+	1:1,024	Pos
B5	–	+	1:4,096	Pos
B6	+	+	1:512	Pos
B7	–	+	1:512	Pos
B8	+	No sample	1:8	Neg
B10	+	+	1:4	Neg
B11	–	+	1:256	Pos
B12	–	–	1:1,024	Pos
B13	–	–	0	Neg
B14	–	+	1:128	Pos
B15	+	+	1:64	Neg
B16	+	+	1:16	Neg
B17	–	+	0	Neg
B18	+	+	1:512	Pos
B19	+	+	1:4	Neg
B20	–	+	1:128	Pos
B21	–	+	1:16	Neg

[a]DFA, direct fluorescent assay; Neg, negative; Pos, positive.

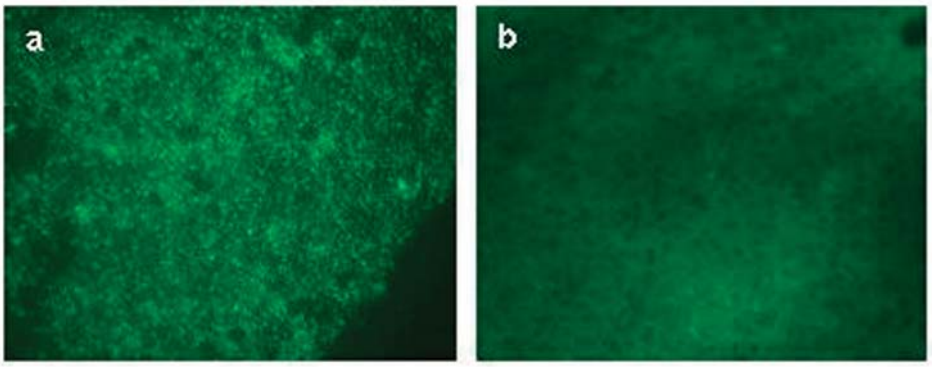

Figure 2. Direct fluorescent assay (DFA) results on spleen tissues from a seronegative (panel a) and seropositive (panel b) prairie dog.

Studies of the black-tailed prairie dog in nature have also documented cannibalism (16). At the Texas exotic animal facility, group A and B animals were placed together in a single metal bin, which allowed unnaturally close contact and conditions. Also, the use of wood chip bedding increased the likelihood that buried carcasses would not be seen, probably contributing to delayed removal of deceased animals, thereby increasing the opportunity for cannibalism.

Since, *F. tularensis* in pet prairie dogs presented an unaddressed public health threat for their owners, we focused our efforts on the recovery of live organisms. We were able to culture infectious bacteria from both dead and live, infected animals. Moreover, our study is the first to provide evidence that prairie dogs can develop antibodies against *F. tularensis*. The seropositive prairie dogs might have survived long-term, since these animals had decreased levels of infecting bacteria and were blood-culture negative (unpub. data). These findings raise the possibility that persistent infection occurs in prairie dogs and suggests a potential role of prairie dogs as reservoirs of *F. tularensis* in nature. Our findings with prairie dogs are very similar to previous reports documenting chronic infection in seropositive voles infected orally with *F. tularensis* (17,18). In one of those studies, seropositive voles were shown to harbor live *F. tularensis* for as long as 313 days.

Although culture is considered the standard criterion for identification, *F. tularensis* is a fastidious organism making culture recovery a challenge, especially when analyzing animal carcasses. Tissues from dead animals are often overgrown with normal flora and other environmental contaminants. Past studies with carcasses have had limited success, and the culture recovery rates were approximately 30% (19). We achieved a culture recovery rate of 89.7% from *F. tularensis*–infected animals (both live and dead animals), demonstrating the sensitivity and usefulness of culture. In light of our findings, we suggest that culture on CHAB media containing antibiotics be attempted more routinely for diagnosis of *F. tularensis* infection in animal and field specimens such as water, mud, and grass or hay.

Additionally, when culture was used for detection of *F. tularensis* in animals that did not die of the disease, we found it more sensitive than either DFA (50%) or serologic testing (50%) and capable of detecting *F. tularensis* in all cases (100%). This high culture recovery rate is probably due to the freshness and relatively uncontaminated state of the specimens used for culture. The comparatively low detection levels of DFA and serologic testing were likely influenced by the fact that these animals were at varying stages of infection (acute phase and convalescent phase), making diagnosis by either DFA or serologic testing less than optimal. This suggests that for surveillance studies of *F. tularensis* infection in wild rodent populations, culture of fresh tissues is the preferred diagnostic method.

In contrast, in detecting *F. tularensis* in animals that died of the disease, DFA was more sensitive than culture (85.1%) and capable of detecting *F. tularensis* in all animals. For fatal cases of tularemia in prairie dogs, the levels of *F. tularensis* were extremely high in both spleen and liver, simplifying identification by DFA. Culture recovery of *F. tularensis* was probably more difficult because of deterioration of the samples and loss of bacterial viability over time. Indeed, *F. tularensis* in the tissues of the seven presumptive-positive animals was noncultivatable and noninfectious as shown by passage of the tissues through Swiss-Webster mice.

Presumably, one or more *F. tularensis*–infected prairie dogs were among the thousands trapped and shipped to the Texas exotic animal facility. On arrival at the facility, the infected prairie dogs died. The bacterium was then transmitted throughout hundreds of prairie dogs at the facility most likely as the result of cannibalism. Several cases (Introduction and 7) of tularemia in prairie dogs have now been documented, suggesting that a proportion of wild prairie dogs harbor live *F. tularensis.* Environmental stresses, such as capture, transit, and crowding, may induce productive infection that manifests as severe

disease and death. Given the seriousness of the resulting disease and the public health risk for pet-to-human transmission, long-term studies are needed to determine the length of time seropositive prairie dogs can harbor live *F. tularensis* and whether they are reservoirs of tularemia in nature.

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Monkeypox Transmission and Pathogenesis in Prairie Dogs

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During May and June 2003, the first cluster of human monkeypox cases in the United States was reported. Most patients with this febrile vesicular rash illness presumably acquired the infection from prairie dogs. Monkeypox virus was demonstrated by using polymerase chain reaction in two prairie dogs in which pathologic studies showed necrotizing bronchopneumonia, conjunctivitis, and tongue ulceration. Immunohistochemical assays for orthopoxviruses demonstrated abundant viral antigens in surface epithelial cells of lesions in conjunctivae and tongue, with lower amounts in adjacent macrophages, fibroblasts, and connective tissues. Viral antigens in the lung were abundant in bronchial epithelial cells, macrophages, and fibroblasts. Virus isolation and electron microscopy demonstrated active viral replication in lungs and tongue. These findings indicate that both respiratory and direct mucocutaneous exposures are potentially important routes of transmission of monkeypox virus between rodents and to humans. Prairie dogs offer insights into transmission, pathogenesis, and new vaccine and treatment trials because they are susceptible to severe monkeypox infection.

During May and June 2003, the first cluster of human monkeypox cases in the United States was reported (1–4). Most human case-patients with this febrile vesicular rash illness were believed to have acquired the infection from prairie dogs (*Cynomys* spp.) that became ill after contact with various exotic African rodents (*Funiscuirus* spp., *Heliosciurus* spp., *Cricetomys* spp., *Atherurus* spp., *Graphiurus* spp., and *Hybomys* spp.) shipped from Ghana to the United States in April 2003 (1,2). Some African rodents from this shipment became ill and died shortly after arriving in the United States. Culture and polymerase chain reaction (PCR) demonstrated monkeypox virus in two rope squirrels (*Funiscuirus* spp.), one Gambian rat (*Cricetomys* sp.), and three dormice (*Graphiurus* spp.) (2). The two prairie dogs described in this report came from the same wholesale pet store where other monkeypox virus–infected rodents were housed.

In areas of Africa where monkeypox infections in humans have been documented previously, serologic surveys of wild animals have suggested that infection with monkeypox virus occurs in several species of African rodents (5). The virus has been isolated from skin lesions on a rope squirrel from Zaire (6). However, pathologic studies of naturally acquired monkeypox virus infections in animals have not been reported. Here we present pathologic, immunohistochemical (IHC), electron microscopy (EM), and molecular findings in two monkeypox virus–infected prairie dogs associated with the recent outbreak of the disease in humans in the United States. These results help elucidate the pathogenesis of naturally occurring monkeypox virus infections in mammals and shed light on possible routes of viral transmission between rodents and to humans during this outbreak.

Materials and Methods

The two prairie dogs from whom data are presented here came from a group of approximately 200 prairie dogs that were housed at a wholesale pet store with multiple species of exotic African rodents. About 110 prairie dogs were sold before 15 reportedly became ill. Of the 15 ill prairie dogs, 10 died rapidly, and 5 exhibited anorexia, wasting, sneezing, coughing, swollen eyelids, and ocular discharge. Initially, tularemia was suspected clinically, and two of the ill prairie dogs were euthanized for pathologic confirmation. The remaining prairie dogs were destroyed (3,4).

Culture and Molecular Analysis

Fresh lung tissue specimens were evaluated for the presence of viable infectious virus by injecting them into

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BSC-40 tissue culture and observing them daily for typical cytopathic effect. Fresh, unfixed tissues from lung were examined for specific signatures of monkeypox virus by using PCR. Samples were initially evaluated by single-gene PCR, followed by restriction-endonuclease fragment length polymorphism (RFLP) identification of monkeypox-specific fragment patterns (7–9). An additional novel multiplex standard PCR assay (10) discriminated monkeypox from vaccinia and variola orthopoxvirus species on the basis of specific DNA polymerase gene amplicons. Real time (RT)-PCR assays included a specific monkeypox virus nucleic acid signature encoded in the envelope gene (monkeypox-B6R) and orthopoxvirus nucleic acid signatures in the DNA polymerase gene (E9L non var.). Controls included DNA from monkeypox virus, other orthopoxviruses, and no-template controls.

Pathologic Examination

Tissues were examined grossly and microscopically. Hematoxylin- and eosin-stained slides were prepared from formalin-fixed, paraffin-embedded samples of the central nervous system, conjunctivae, tongue, salivary glands, lungs, heart, liver, gastrointestinal tract, spleen, adrenal glands, kidneys, and lymph nodes. IHC assays were performed as previously described for other infectious agents in the DAKO autostainer (Dako Corp., Carpinteria, CA) (11–14). Briefly, 3-µm sections of the tissues were deparaffinized and rehydrated. Tissue sections were then digested with 0.1 mg/mL proteinase K (Roche Diagnostics, Indianapolis, IN) in 0.6 M Tris (pH 7.5)/ 0.1% $CaCl_2$ (proteinase K buffer) for 15 min and later blocked with 20% normal sheep serum in Tris-saline-tween-20. Tissue sections were incubated for 60 min with a primary antibody. Primary antibodies included three polyclonal antiortho-poxvirus antibodies (rabbit antivariola virus, mouse antivaccinia virus, and rabbit antimonkeypox virus [Centers for Disease Control and Prevention (CDC), Atlanta, GA]); in addition, a monoclonal anti-*Francisella tularensis* antibody (Naval Biodefense Program, Bethesda, MD), and a polyclonal anti–*Yersinia pestis* antibody (CDC, Fort Collins, CO). This was followed by sequential application of swine antimouse or swine antirabbit link antibody, avidin-alkaline phosphatase, and naphthol/fast red substrate (Dako Corp.). Sections were then counterstained in Meyer's hematoxylin (Fisher Scientific, Pittsburgh, PA).

Positive controls included formalin-fixed, paraffin-embedded cells infected with variola virus, vaccinia virus, and monkeypox virus. Negative controls included similar cells infected with influenza A virus and human herpesvirus 1 (herpes simplex) and 3 (varicella-zoster); animal tissue samples infected with Ebola virus, *Y. pestis*, and *F. tularensis*; human skin lesions known to have human herpesvirus 1 and 3; and human skin samples with noninfectious dermatitis caused by poison ivy or drug eruptions. Negative controls for the prairie dogs specimens consisted of sequential tissue sections incubated with normal rabbit or mouse serum.

Electron Microscopy (EM)

Specimens for EM were excised from paraffin-embedded blocks of lung and tongue in areas that corresponded to positive IHC results. Tissues were deparaffinized for 1 h in xylene warmed to 60°C, rehydrated through a graded series of alcohols, postfixed in phosphate-buffered 2.5% glutaraldehyde and 1% osmium tetroxide, stained with 4% uranyl acetate, dehydrated through a graded series of alcohols and propylene oxide, and embedded in a mixture of Epon-substitute and Araldite. Ultrathin sections were stained with 4% uranyl acetate and Reynold's lead citrate.

Results

Gross examination of both animals revealed yellow mucoid discharge in the eyelids. One animal had a 3- to 4-mm ulcer in the center of the tongue. The lungs showed patchy areas of red-brown consolidations involving about 50% of the pulmonary parenchyma. The livers were red with a few scattered, tanned, mottled areas. Typical orthopoxvirus sequences were revealed in lung tissue samples by use of a novel multiplex PCR assay, which detected the essential DNA polymerase gene; however, the standard single-gene PCR and RFLP analysis did not show the presence of monkeypox virus. Specific monkeypox virus sequences were also obtained with more sensitive RT-PCR assays. Viral cytopathic effects were observed at days 4 and 5 in cultures inoculated with fresh lung samples.

Histopathologic examination of the eyelids showed a necrotic, ulcerated lesion of the palpebral conjunctiva. The ulcer bed consisted of necrotic debris and pyknotic epithelial cells. Columnar epithelial cells surrounding the ulcer were swollen and contained dense, eosinophilic, cytoplasmic granules of various sizes that suggested Guarnieri-like inclusions. The submucosa showed mixed inflammatory cell infiltrate, necrosis, and edema. Other areas of the palpebral conjunctivae and skin showed inflammatory foci in the epithelium without ulcer formation but with ballooning degeneration of epithelial cells, acantholysis, and occasional cell necrosis. Abundant orthopoxvirus antigens were detected in areas with grossly and microscopically identified lesions (Figure 1A, B) by using orthopoxvirus IHC assays. Viral antigens were present prominently in the squamous and columnar epithelium (Figure 1B) and in lesser amounts in fibroblasts and histiocytes in the ulcer bed or underlying the lesions. In epithelial cells, antigens

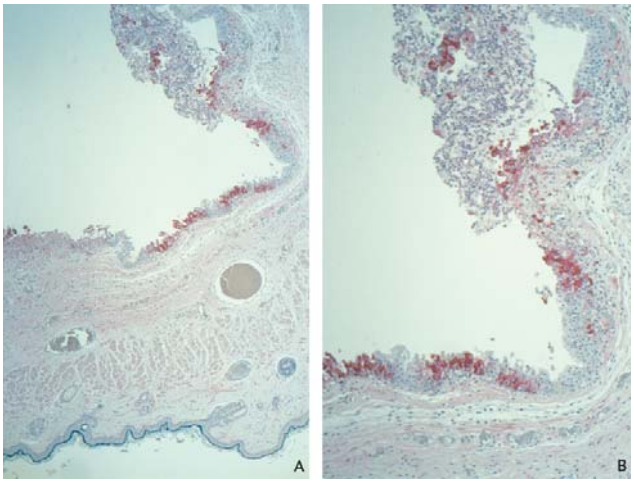

Figure 1. Immunohistochemical staining of a prairie dog eyelid infected with monkeypox virus, showing orthopox virus antigen staining of the cytoplasm of the epithelium of the palpebral conjunctivae (assay using anti–variola virus antibody; original magnifications: A, 12.5X; B, 25X).Note: For full reproduction of these images, please see http://www.cdc.gvo/ncidod/EID/vol10no3/03-0878.htm

were observed in the cytoplasm intensely staining the cytoplasmic Guarnieri-like inclusions.

Histopathologically, the tongue ulcer demonstrated necrosis and mixed inflammation at the ulcer bed (Figure 2A). Nonulcerated mucosa showed focal areas of lichenoid interface, mixed inflammatory infiltrate with necrosis, ballooning degeneration, and dense eosinophilic cytoplasmic granules (Guarnieri-like inclusions) in the squamous epithelium. IHC assays showed viral antigens only in lesions (Figure 2B); the antigens had a pattern similar to that described for the necrotic, ulcerated lesion of the conjunctivae. EM examination revealed abundant mature and immature poxvirus particles in the cytoplasm of epithelial cells (Figure 2C, D).

The lungs showed concentric, coalescing bronchioalveolar pneumonia. Airways showed necrosis and mixed infiltrate of neutrophils and histiocytes in the lumen and epithelium, and the bronchial epithelium showed a reactive proliferative response (Figure 3A). Inflammation extended through the bronchilolar walls into surrounding alveoli, which demonstrated fibrinous edema, necrosis, and marked infiltrate of macrophages, some having intranuclear cytoplasmic inclusions, while others showed multinucleation. Adjacent arterioles showed reactive fibrinocellular edema in the adventitia and inflammatory infiltrate. The nonnecrotic areas of the lung demonstrated intraalveolar edema. IHC assays demonstrated abundant viral antigens in the areas with bronchioalveolar inflammation (Figure 3D). Viral antigens were observed in the cytoplasm of macrophages, bronchial epithelial cells, and fibroblasts; viral antigens were also present in the necrotic debris and interstitial connective tissue (Figure 3C). Immature and mature poxvirus particles were demonstrated inside bronchial epithelial cells by using EM (Figure 3B).

Except for mild portal inflammation in the liver and reactive hyperplasia in the spleen, no significant pathologic changes were noted in other organs. Viral antigens were not observed in other tissues, with the exception of occasional medullary and subcapsular sinusoidal histiocytes in a submandibular lymph node. No IHC evidence of *F. tularensis* and *Y. pestis* was observed in the tissues.

Discussion

During the 2003 outbreak of human monkeypox in the United States, a shipment of African rodents that contained Gambian rats and dormice is thought to have resulted in secondary infection of prairie dogs (1–4). Exposure to infected prairie dogs resulted in 37 human infections involving exotic pet dealers, pet owners, and veterinary

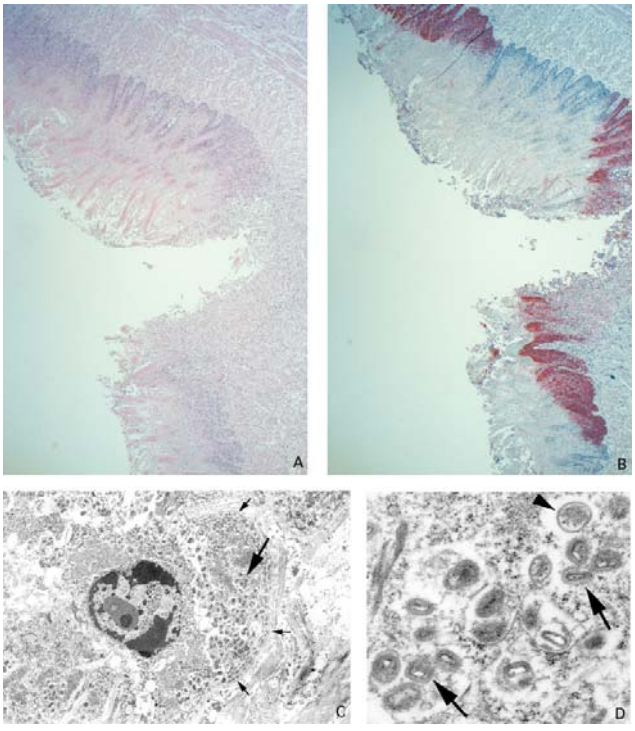

Figure 2. Ulcer on tongue of a prairie dog infected with monkeypox virus (A: hematoxylin and eosin stain, 12.5X original magnification). Orthopox viral antigens are abundant in the squamous epithelium, with lesser amounts in the ulcer bed (B: immunohistochemical stain using the anti-variola virus antibody, 12.5X original magnification). Tongue epithelial cell adjacent to epidermal basement membrane (small arrows) with Guarnieri-like inclusion (large arrow) (C: transmission electron microscopy, 2,400X original magnification). Higher magnification of the Guarnieri-like inclusion shows intracellular immature (arrowhead) and mature (arrows) orthopox virions. The mature virions consist of a dense core surrounded by several laminated zones and enclosed within an outer membrane (D: transmission electron microscopy, 17,000X original magnification). Note: For full reproduction of these images, please see http://www.cdc.gvo/ncidod/EID/vol10no3/03-0878.htm

care workers in the United States (1–3). The mode of transmission of the monkeypox virus between infected animals and humans is not clearly defined, partly because histopathologic and immunohistochemical studies of animals with naturally acquired infection have not been published. The prairie dogs in this study demonstrated abundant viral antigens and mature poxvirus particles in the tongue and conjunctival lesions; hence, direct contact with saliva or exudates from these lesions could have inoculated monkeypox virus to skin or mucous membranes of other hosts. In addition, the lungs demonstrated abundant replicating monkeypox virus in the bronchi and lung parenchyma; thus, transmission to other rodents and humans may have occurred when the infected animal coughed and dispersed infective droplets. Furthermore, the pneumonic process in these prairie dogs suggests a respiratory route of infection between rodents. Thus, the pathologic study of severely ill prairie dogs in this outbreak provided evidence that direct mucocutaneous contact and respiratory routes played a role in transmission, as has been suggested in African outbreaks of human disease (15–19).

Prairie dogs may be an excellent animal model for the further study of monkeypox infections because they are small, plentiful, and susceptible to severe monkeypox virus disease. In naturally or experimentally infected animals, a spectrum of clinical illness will develop; for example, of the nonhuman species that naturally acquire monkeypox virus infections, skin lesions have only been observed in some African primate species and rope squirrels (*Funiscuirus* spp.) (5,20). The pathologic features observed in prairie dogs, including a necrotizing bronchopneumonia, have been described in *Cynomolgus* monkeys infected experimentally by inhalation of monkeypox virus (21). In these animals, the lower respiratory epithelium was the target for primary replication of virus. Monocytes carried the virus to lymphoid tissues, where a secondary viral replication occurred and resulted in the seeding of other tissues, including skin, oral mucosa, gastrointestinal tract, and the tissues of the reproductive system. In this monkey model, secondary viral replication sites had necrotizing lesions. Necrotizing lesions with viral antigens in lymphoid tissues have been seen in other animals, including prairie dogs that fell ill and died during the U.S. outbreak (data not shown). The prairie dogs in this study did not have necrotizing lymphadenitis or splenitis, which may indicate that these rodents were euthanized relatively early in the disease course.

Monkeypox virus infected predominantly epithelial cells in conjunctivae, tongue, and bronchi. Histopathologically, infected epithelial cells showed prominent ballooning degeneration and dense, eosinophilic, cytoplasmic granules that were difficult to distinguish from keratohyalin bodies. Epithelial cells occasionally coalesced, forming syncytia, and their nuclei showed eosinophilic, ground-glass staining that must be differentiated from herpetic inclusions for diagnostic purposes. By use of IHC, the eosinophilic cytoplasmic granules seen in infected epithelial cells were proven to be viral inclusions (Guarnieri-like inclusions), and EM examination corroborated these findings. In the prairie dogs studied, orthopoxvirus antigens were also demonstrated in other cells, including macrophages and fibroblasts in areas adjacent to infected epithelial cells. Histopathologic studies of human monkeypox skin vesicular lesions showed an IHC staining pattern similar to that found in the tongue and conjunctiva of the infected prairie dogs (1–4,22,23).

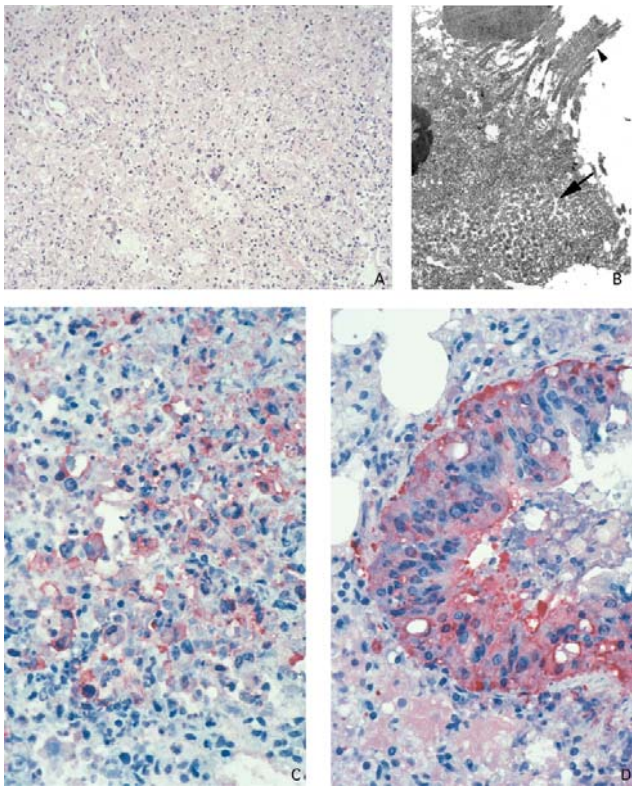

Figure 3. Lung of prairie dog infected with monkeypox virus, showing abundant intraalveolar mixed inflammatory infiltrate and necrosis (A: hematoxylin and eosin stain, 50X original magnification). Orthopox viral antigens are abundant in the cytoplasm of the bronchiolar epithelium (D: immunohistochemical assay anti–variola virus antibody, 100X original magnification). Macrophages, fibroblasts, and alveolar epithelial cells, as well as necrotic debris demonstrate orthopox viral antigens in pneumonic areas of the lung (C: immunohistochemical stain anti–variola virus antibody, 100X original magnification). Accumulation of intracellular mature virions (arrow) in bronchial epithelial cell (arrowhead pointing to cilia) (B: transmission electron microscopy, 2,400X original magnification). Note: For full reproduction of these images, please see http://www.cdc.gvo/ncidod/EID/vol10no3/03-0878.htm

The U.S. monkeypox virus outbreak demonstrated how new diseases can emerge due to facile movement of species from one location to another (including the illegal trans-

porting of species). The investigation of human cases at the wholesale pet store that housed a variety of African rodents and the two prairie dogs studied revealed a spectrum of monkeypox-associated disease ranging from only serologic evidence of monkeypox infection to febrile vesicular rash illness (3). Differences in disease severity may relate to the source of exposure, transmission route (i.e., inhalational versus direct mucocutaneous contact), amounts of virus inoculated, virus strain, or host susceptibility. Epidemiologic studies of human monkeypox infections have shown that younger children and persons not vaccinated against smallpox can have severe disease and complications, which supports the importance of host susceptibility, including previous immunity (18,24,25). This outbreak of monkeypox virus infection in humans and nonhuman animals is an important reminder to monitor surveillance programs for febrile rash illnesses designed to detect potential bioterrorism attacks with smallpox virus, which may be beneficial for detecting emerging infections (26).

A variety of methods were used to diagnose and study monkeypox virus infection in these prairie dogs. IHC studies permitted demonstration of the virus in the context of histopathology. EM and culture demonstrated viral replication, while molecular studies were essential for determining the specific signatures of monkeypox virus. In the prairie dogs studied, standard PCR and RFLP did not show monkeypox DNA. However, RT-PCR detected monkeypox viral DNA since RT-PCR is more sensitive and can be used to accurately titrate up to 4–10 DNA copies (27).

Studying necropsied animal specimens was of great benefit during this monkeypox outbreak investigation. Research into the natural biology of monkeypox has been limited because the disease is rare in humans and no descriptions exist of naturally acquired animal infections. The pathologic findings in this study of prairie dogs can be used to better define possible transmission routes and pathogenesis of human and animal monkeypox, and such a model may help develop new vaccine and treatment strategies for orthopoxvirus infections.

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Acute Spotted Fever Rickettsiosis among Febrile Patients, Cameroon

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Although potential arthropod vectors are abundant in Cameroon, acute febrile illnesses are rarely evaluated for arboviral or rickettsial infections. Serum samples from 234 acutely febrile patients at clinics in Tiko and Buea, Cameroon, were examined for antibodies to *Rickettsia africae* and African alphaviruses and flaviviruses. These serum samples did not contain antibodies against typhoid, and blood malarial parasites were not detected. Serum samples of 32% contained immunoglobulin M antibodies reactive with *R. africae* by immunofluorescence assay and were reactive with outer membrane proteins A and B of *R. africae* by immunoblotting. These findings established a diagnosis of acute rickettsiosis, most likely African tick-bite fever. Hemagglutination inhibition testing of the serum samples also detected antibodies to chikungunya virus (47%) and flaviviruses (47%). High prevalence of antibodies to arboviruses may represent a major, previously unrecognized public health problem in an area where endemic malaria and typhoid fever have been the principal diagnostic considerations.

Cameroon is situated 2°–14° north of the equator; it has a vast tropical rain forest located 2°–5° north of the equator, which provides a good habitat for a variety of hematophagous arthropods. Vector-borne bacterial and viral diseases are rarely considered by local clinicians, whose primary diagnostic focus is on endemic malaria and typhoid fever. Chikungunya fever is an arboviral disease transmitted by mosquitoes of the genus *Aedes*; it occurs in neighboring Nigeria; and epidemics have been reported in Angola, Burundi, the Central African Republic, Kenya, Namibia, Senegal, South Africa, Tanzania, Uganda, and Zimbabwe (1–3). Antibodies to chikungunya virus (CHIKV) also were observed in German aid workers who had served in Bénin, Burkina Faso, and Zambia (4). The extent of CHIKV infection in the human population of Cameroon is unknown.

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Aedes aegypti and *A. albopictus*, the major vectors of dengue fever, are both present in Cameroon (5,6). Although no information is available on the prevalence of dengue fever in Cameroon, epidemics of the disease have been reported in other neighboring African countries. Epidemic dengue hemorrhagic fever has not been reported in Africa, but sporadic cases clinically compatible with it have been reported in Mozambique and Djibouti (7). Yellow fever is endemic in much of sub-Saharan Africa, and large outbreaks of the disease have been reported in Ethiopia, Senegal, Nigeria, and Guinea (8,9). Although epidemics of yellow fever have not been reported in Cameroon, it is nonetheless considered to be a high-risk zone for the disease (8). Currently, no immunization programs are in place in the country to prevent yellow fever. Febrile illnesses such as chikungunya fever, dengue fever, and nonicteric yellow fever can be difficult to recognize, especially during the early stages of the disease and in a malaria-endemic zone (2).

Rickettsia africae is a spotted fever group rickettsia transmitted by *Amblyomma* ticks (10). It is endemic in some southern African countries, such as Zimbabwe and South Africa; most cases are reported in travelers returning from these countries (11,12). Although serologic surveys in Angola, Burkina Faso, Central African Republic, Congo, Ivory Coast, Mali, and Zimbabwe have previously detected antibodies to spotted fever group rickettsiae, the first human case of *R. africae* infection was not reported until 1992 (13–15). An earlier serologic survey that used a method less reliable than the immunofluorescent antibody assay demonstrated rickettsial antibodies in cattle and humans in the northern region of Cameroon and in other animals in the south of the country (16,17). Since then, little has been done to determine the incidence of rickettsioses in Cameroon. In this study, we sought to detect antibodies to spotted fever group rickettsiae, CHIKV, yellow fever, dengue, West Nile, and Spondweni viruses in serum samples collected from patients with symptoms of an acute febrile illness seen at clinics in the South West

Province of Cameroon but in whom laboratory results for malaria and typhoid fever were negative.

Materials and Methods

Study Population

A total of 234 serum samples were obtained at the Cameroon Development Corporation Central Clinic in Tiko (180 samples) and the Mount Mary Health Center in Buea (54 samples) from February 15 to March 31, 2001, from patients with clinical symptoms of a febrile illness and laboratory test results that excluded a diagnosis of malaria or typhoid fever. The samples were collected into sterile containers, and the serum samples were stored at –20°C. One hundred forty-two samples were from female patients, and 92 were from male patients; most samples were from adults (Table 1). The study participants were all residents of the South West Province of Cameroon from locations along the Atlantic Coast (Figure 1): Buea (4° 9′ N, 9° 13′ E), 52 patients; Limbe (4° 1′ N, 9° 12′ E), 27 patients; Muyuka (4° 10′ N, 9° 25′ E), 22 patients; and Tiko (4° 2′ N, 9° 19′ E), 133 patients. Yellow fever vaccine has not been administered routinely or in response to disease outbreaks in this region. The research protocol was approved by the Cameroon Ministry of Health and the administration of the clinics to ensure the ethical conduct of the study.

Immunofluorescence Assay

Immunofluorescence assays (IFAs) were performed to detect antibodies to *R. africae* and *R. conorii*, as previously reported (18). Serial twofold dilutions (1 of 32 to 1 of 4,096) of human serum were prepared in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% Tween 20. Antigen slides were blocked in PBS containing 1% BSA and 0.01% sodium azide. Ten microliters of each serum dilution was added to each well of the antigen slide and incubated for 30 min at 37°C in a humidity chamber. The slides were subsequently rinsed with a stream of PBS containing 0.1% Tween and then washed twice more in the same solution for 10 min. Fluorescein isothiocyanate–conjugated goat anti-human immunoglobulin (Ig) A, IgG, and IgM immune serum (KPL Inc., Gaithersburg, MD), 10 µL diluted 1 to 100 in PBS containing 1% BSA and 0.01% Tween 20, was added to each well and incubated in a humidity chamber for 30 min at 37°C. The slides were washed once in PBS containing 0.1% Tween 20 for 10 min and once with PBS containing 0.1% Tween 20 and 0.01% Evans blue for 10 min. The rinsed slides were blot dried, mounted with gel mount (Biomeda Corp, Foster City, CA) and observed under a fluorescence microscope at 400x magnification. Serum samples yielding distinctly fluorescent rickettsiae at a 1 to 64 dilution or higher were considered positive.

Table 1. Distribution of febrile Cameroonian patients in this study according to age and sex

Age group (y)	Female	Male	Total
≤5	2	2	4
6–20	13	10	23
≥21	127	80	207
Total	142	92	234

For assaying IgM antibody to *R. africae* and *R. conorii*, IgG was removed from IgG/IgA/IgM–positive serum by using recombinant protein G (MiniRapiSepM, PanBio, InDx, Baltimore, MD) according to the manufacturer's instructions. Serum samples with specific fluorescence at ≥1:32 dilution were considered positive. Serial twofold dilutions of serum were tested to determine the endpoint titer.

Western Immunoblot Assay

R. africae and *R. conorii* were released from Vero cell components by sonication and purified by density gradient centrifugation. Proteins were examined for immunoreactivity with the Cameroonian sera by Western immunoblotting (19,20).

Preparation of Arbovirus Antigens

Arbovirus antigens were produced by sucrose-acetone extraction (21) of brains from newborn mice inoculated intracerebrally with the following arboviruses: CHIKV (Bili 4 strain), o'nyong-nyong virus (ONNV) (MP 30), yellow fever virus (17D), dengue 1 virus (Mochizuki strain), dengue 2 virus (New Guinea C), dengue 3 virus (H87), dengue 4 virus (H-241), West Nile virus (NY 385/99), and Spondweni virus (SAAR94). All arboviruses were obtained from the Arbovirus Reference Center at the University of Texas Medical Branch.

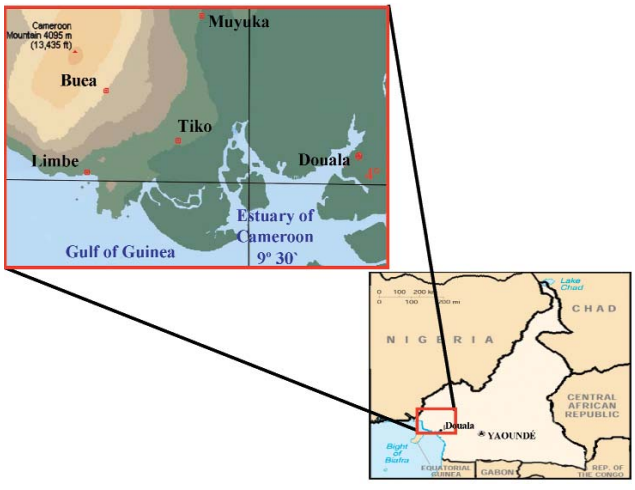

Figure 1. Location of townships in South West Province of Cameroon where samples were obtained.

Hemagglutination-Inhibition Test

Hemagglutination-inhibition (HI) tests were performed by using a microtechnique described previously (21). Before being tested, serum samples were treated with acetone to remove nonspecific inhibitors, and then goose erythrocytes were added to absorb natural agglutinins. CHIKV and ONNV antigens were tested at pH 6.2; dengue 1, 2, and 3 and yellow fever viruses were tested at pH 6.4; and dengue 4, West Nile, and Spondweni viruses were tested at pH 6.6. For the HI test, 4–8 U of antigen was used. Titers were recorded as the highest dilutions causing complete or almost complete inhibition of hemagglutination.

Complement Fixation Test

Complement fixation (CF) tests were performed according to a microtechnique modified from Fulton and Dumbell (22), using 2 full U of guinea pig complement and the sucrose-acetone–extracted CHIKV and ONNV antigens described earlier. Serum samples were incubated at 60°C for 20 min before testing. Titers were recorded as the highest dilution giving 3+ or 4+ fixation of complement on a scale of 0 to 4+ (0 = complete hemolysis and 4+ = no hemolysis).

Results

Serologic Testing for Rickettsial Antibodies

The 234 serum samples initially were screened by IFA for IgG, IgA, and IgM antibodies against *R. africae* and *R. conorii*; the samples containing antibodies at a titer of 32 were examined by the IgM-specific IFA. The results indicated that 75 patients (32.1%) had IgM antibodies reactive with *R. africae*. Fifty-one (35.9%) of the 142 female patients had IgM antibodies against *R. africae*; and 24 (26.1%) of the male patients had IgM anti–*R. africae* antibodies. The distribution of endpoint IgM titers for *R. africae* was 32 (24%), 64 (18%), 128 (20%), 256 (14.4%), 512 (12%), 1,024 (8%), and 2,048 (2.7%).

The distribution of IgM titers against *R. conorii* among the 75 *R. africae*–positive serum samples was 32 (29.3%), 64 (32.8%), 128 (17.2%), 256 (19%), and 512 (1.7%). Twenty-six of the samples had antibody titer fourfold or greater for *R. africae* than for *R. conorii*. None of the serum samples had titers fourfold higher for *R. conorii.* Nineteen serum samples had antibody titers twofold higher for *R. africae*; three had antibody titers twofold higher for *R. conorii*. Eight serum samples had a titer of 32 for *R. africae* and were negative for *R. conorii.* Nineteen samples had the same titers for *R. africae* and *R. conorii*. Of the patients with IgM antibodies to spotted fever group rickettsiae, 67 (89%) were adults, and 8 (11%) were <20 years of age. None of four children <5 years of age had IgM antibodies to *R. africae.* Western immunoblot analysis confirmed the IFA results (Figure 2). The reactive serum samples contained antibodies that were immunoreactive with both OmpA and OmpB of *R. africae*.

Serologic Testing for Arboviral Antibodies

HI testing of serum samples from febrile patients in South West Province demonstrated antibodies to CHIKV, dengue 1–4, yellow fever, West Nile, and Spondweni viral antigens (Table 2). Antibodies were detected in some of the serum samples for all of the viruses tested, and considerable cross-reactivity among the flaviviruses was observed. The HI tests with dengue 1–4 viruses and yellow fever virus antigens yielded similar results; titers ranged from 20 to 2,560. HI antibodies to CHIKV antigen were detected in 103 (44%) of the 234 serum samples, and a titer of ≥1,280 was observed in 11 samples. Comparative CF testing demonstrated that the endpoint titers were higher against CHIKV antigen than ONNV antigen, a finding that suggests that CHIKV was the infecting alphavirus.

Eighty-four (35.9%) of the 234 serum samples contained HI antibodies reactive with one or more of the dengue 1–4 viruses, 93 (39.7%) had antibodies to yellow fever virus, 62 (26.5%) contained antibodies against West Nile virus, and 65 (27.8%) had antibodies to Spondweni virus. In all, 110 (47%) of the serum samples contained antibodies to one or more of the flaviviruses. Antibodies to CHIKV and the flaviviruses were detected in patients from each of the study locations (Table 2).

Discussion

In Cameroon, as in many other African countries, rickettsioses and arboviral infections are rarely considered when evaluating patients with acute, undifferentiated febrile illnesses. This situation can be attributed in part to unavailability of specific laboratory tests, equipment, and expertise and also the limited economic resources in many countries of the region. In Cameroon, most patients are evaluated by clinical laboratory methods only for malaria and typhoid fever. Since many patients with rickettsial and arboviral ill-

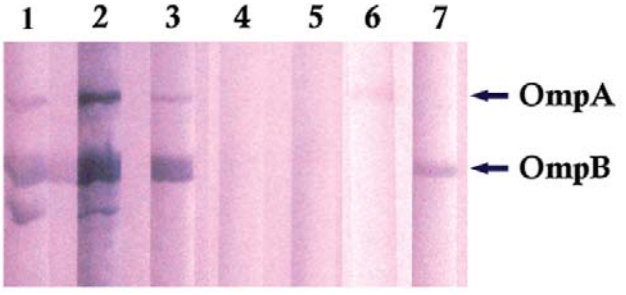

Figure 2. Immunoblot of *Rickettsia africae* antigens with *R. africae*-positive and -negative patient serum samples. Lanes 1-3: *R. africae*-positive patients' serum samples; lanes 4-5: *R. africae*-negative patients' serum samples; lane 6: anti-OmpA monoclonal antibody; lane 7: anti-OmpB monoclonal antibody.

Table 2. Geographic origin of 234 Cameroonian patients with antibodies to chikungunya, yellow fever, dengue, West Nile, and Spondweni viruses[a]

Antigen	Buea (n = 52)	Limbe (n =27)	Muyuka (n = 22)	Tiko (n = 133)	Total	Geometric mean titer[b]
Chikungunya	15	18	5	65	103	250
Yellow fever	12	10	4	67	93	56
Dengue 1	5	11	5	59	80	91
Dengue 2	6	9	5	55	75	66
Dengue 3	6	6	3	41	56	71
Dengue 4	4	6	3	42	55	85
West Nile	0	9	2	51	62	71
Spondweni	0	9	3	53	65	77

[a]As detected by hemagglutination inhibition test.
[b]Reciprocal of titers.

nesses initially have acute febrile syndromes, their diagnosis is difficult without confirmatory laboratory tests.

Previous retrospective serologic surveys detected antibodies to rickettsiae in Zimbabwe, Ivory Coast, Central African Republic, and Egypt (15,23–26). However, detecting IgM antibodies to rickettsiae by IFA, in association with febrile illness, is a better approach to identifying recent or current infections; Western immunoblot assay can be used to confirm positive IFA results.

Our results indicate that one or more rickettsioses are endemic in Cameroon. Most previous serologic surveys have detected antibodies in healthy study participants (14,17,18,26,27); however, our study population was composed of patients with acute febrile illnesses other than malaria or typhoid fever. The specific cause of the agent that stimulated the antibodies to spotted fever group rickettsiae in these patients remains to be determined. Raoult et al. (11) reported spotted fever rickettsiosis in travelers returning to Europe from Africa with documentation of *R. africae* infections by isolation of the agent and specific polymerase chain reaction results. The same authors also evaluated serologic methods to distinguish between infection with *R. africae* and *R. conorii*; they reported that a fourfold or greater titer to *R. africae* by IFA had a specificity of 100% but a sensitivity of only 26% (11). This criterion supported the diagnosis of *R. africae* infection in 26 (35%) of the 75 patients in our study with acute rickettsiosis and did not establish a diagnosis of *R. conorii* infection in any patient. Our immunoblot detection of antibodies to *R. africae* OmpA and OmpB also supported the diagnosis of *R. africae* infection rather than *R. conorii*. However, cross-reactivity of the antibodies with OmpA of another rickettsial organism that was not included in the study, including any undiscovered rickettsiae, is possible. Raoult et al. (11) have diagnosed *R. africae* infections in individual travelers whose infections were acquired in nearby Central African Republic and Gabon. Although our findings suggest that the infections in Cameroonian patients were caused by *R. africae*, the possibility of the occurrence of another spotted fever group rickettsial infection cannot be excluded because spotted fever group rickettsiae share closely related antigens. Moreover, the most reliable and specific method to establish the identity of the causative agent is isolating it from the blood or tissue of suspected patients; this remains to be achieved in Cameroon. Other problems that need to be addressed include a full description of the clinical spectrum of this rickettsiosis in African patients, the risk factors for severe illness, the vector(s), natural history of the bacterium, and epidemiology of the disease.

After an incubation period averaging 6–7 days, *R. africae*–infected travelers returning to Europe and North America manifested an influenza-like syndrome including fever (88%), myalgia (63%), eschars (95%), regional lymphadenopathy (43%), and rash (46%) in one series (11). A subsequent series of Norwegian travelers to sub-equatorial Africa had a similar syndrome of fever, headache, and myalgia but with a lower proportion of patients with an eschar (53%) (28). The low proportion of children in our study suggests that severity of the clinical manifestations may be age-dependent, as has been documented for Rocky Mountain spotted fever and louse-borne typhus fever (29). To date, African tick-bite fever has been characterized as a mild illness. However, the presence of glucose-6-phosphate dehydrogenase deficiency and the empiric treatment of the febrile illness with sulfonamide antimicrobials, both frequent situations in Africa, might result in more severe disease; this possibility needs to be evaluated (30–33). It could be predicted that *A. variegatum* might transmit *R. africae*, and that *Rhipicephalus* species would transmit *R. conorii*; but the susceptibility of other ticks in Cameroon to these rickettsiae has not been studied. The unexpectedly high incidence of spotted fever rickettsiosis in this population also suggests that the course of illness in many febrile patients in Cameroon might be ameliorated by early treatment with an antirickettsial drug such as doxycycline.

CHIKV infection is common in sub-Saharan Africa; antibodies to CHIKV have frequently been detected during serosurveys throughout the humid forest and semi-arid savannas of Africa (1,2,34–36). Although CHIKV and ONNV are closely related (34), our serum samples contain-

ing antibodies to alphaviruses yielded much lower titers against the ONNV antigen, suggesting that cross-reactivity was at a low level and that CHIKV was the circulating agent. Chikungunya fever is characterized by fever, headache, nausea, vomiting, myalgia, rash, and arthralgia (35). These clinical symptoms are similar to those of dengue viral infection and can lead to misdiagnosis (37). Evidence suggests that CHIKV circulates continually in sylvatic cycles in Africa; the virus has been isolated from forest-dwelling mosquitoes in several African countries including Senegal, Ivory Coast, and South Africa (35,36).

Dengue fever is endemic in tropical and subtropical regions worldwide. The possibility that Cameroon is another dengue-endemic region would not be surprising. This infection, which usually manifests as undifferentiated fever, can lead to hospitalization of large numbers of people. Outbreaks cause illness and death rates with substantial socioeconomic impact. The results of our study indicate that rickettsial and arboviral infections are common among residents of Cameroon and that local health personnel should include them in their differential diagnosis. For both the arboviral and rickettsial agents, much work remains to be done, particularly identification of the viruses and rickettsiae in patients and arthropods.

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Genomic Changes of Chagas Disease Vector, South America

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We analyzed the main karyologic changes that have occurred during the dispersal of *Triatoma infestans*, the main vector of Chagas disease. We identified two allopatric groups, named Andean and non-Andean. The Andean specimens present C-heterochromatic blocks in most of their 22 chromosomes, whereas non-Andean specimens have only 4–7 autosomes with C-banding. These heterochromatin differences are the likely cause of a striking DNA content variation (approximately 30%) between Andean and non-Andean insects. Our study, together with previous historical and genetic data, suggests that *T. infestans* was originally a sylvatic species, with large quantities of DNA and heterochromatin, inhabiting the Andean region of Bolivia. However, the spread of domestic *T. infestans* throughout the non-Andean regions only involved insects with an important reduction of heterochromatin and DNA amounts. We propose that heterochromatin and DNA variation mainly reflected adaptive genomic changes that contribute to the ability of *T. infestans* to survive, reproduce, and disperse in different environments.

American trypanosomiasis or Chagas disease is well recognized as the most serious human parasitic disease of the Americas in terms of its social and economic impact (1). This disease is caused by the flagellate protozoan *Trypanosoma cruzi*, and it is transmitted by blood-sucking insects of the subfamily Triatominae (Hemiptera, Reduviidae). There is no vaccine against *T. cruzi*; therefore, disease control relies on eliminating domestic vector populations by spraying infested houses with residual insecticides.

The epidemiologic importance of Chagas disease vectors largely depends on the vectors' spreading ability and adaptation to domestic habitats. Therefore, studies on the changes that have taken place in such domestication and geographic expansion may contribute to understanding the basic process by which some species of Triatominae invade new habitats and colonize human habitations. These analyses are fundamental in the design of control campaigns because their results will help determine the most appropiate strategy for insecticide application. Knowledge of the genetic structure of insect populations (including the evaluation of gene flow between domestic and sylvatic populations), as well as their domestication and spreading capabilities, are essential tools for effective vector control (2).

Triatoma infestans represents the best example of spreading and adaptation to domicilies observed in a triatomine species. This species is the main and widespread vector in South America, responsible for about half of the 12 million cases of Chagas disease reported worldwide. Although its distribution is now being substantially reduced by large-scale control interventions within the Southern Cone Initiative, launched in 1991 by six South American countries (1), its distribution in the mid-1980s was very wide, including vast regions of Argentina, Bolivia, Brazil, Chile, Paraguay, southern Peru, and Uruguay (Figure 1). *T. infestans* is found almost exclusively in domestic and peridomestic environments, occupying cracks and crevices in rural dwellings and domestic animal enclosures. The presence of this species in sylvatic habitats (rock piles in association with wild guinea pigs) has only been confirmed in the Andean valleys of Cochabamba and Sucre in Bolivia (3–5). This finding, together with historical reconstruction (6) and genetic analyses (7), suggests that central Bolivia may be the site of origin and dispersal of *T. infestans* throughout South America.

One important approach used to establish genetic variation in *T. infestans* is cytogenetic analysis. The diploid chromosome number of *T. infestans* is 22, including 10 pairs of autosomes and 1 pair of sex chromosomes (XY in males, XX in females) (8). The three large autosomal pairs and the Y chromosome present C-heterochromatic blocks

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Figure 1. Location of the collection sites of *Triatoma infestans* analyzed in this study. Dotted lines indicate *T. infestans* distribution during the 1980s. Full circles indicate Andean samples. Open squares indicate non-Andean samples. (See number identification of each population in Tables 1–3.)

(9). Based on a great variation in the quantity and position of these C-heterochromatic regions, an extensive polymorphism in natural populations from Uruguay has been described (10). This variation in the three large autosomal pairs was also described in laboratory-reared specimens from Brazil, Paraguay, Argentina, and Chile (11,12).

We present an extensive analysis, including specimens from several countries (Figure 1). Using flow cytometry for DNA quantification and C-banding technique, we have determined the karyologic changes that have occurred during the dispersal of *T. infestans*. This analysis has allowed us to identify two very different chromosomal groups and to discuss the role of heterochromatin and genome size variation in the karyologic evolution of *T. infestans*.

Materials and Methods

Material Analyzed

A total of 209 *T. infestans* specimens from natural populations were examined by C-banding (Tables 1 and 2, Figure 1). Currently, several of these populations, particularly from Uruguay and Brazil, have disappeared as a result of intensive control interventions. Five male specimens of the experimental progeny obtained by crossing insects from Brazil and Bolivia (Cochabamba Valley) were also examined. Flow cytometric analysis of DNA content was performed in 42 male insects. Student *t* test was used for statistical analysis of the results obtained by C-banding and flow cytometry; $p < 0.001$ was considered significant.

Chromosome Preparations and Banding Procedures

Gonads (testes and ovaries) from adult insects (occasionally fifth-stage nymphs) were removed, fixed in ethano–acetic acid (3:1), and stored at -20°C. C-banding treatment was carried out on air-dried squash, as previously described (13).

The C-banding pattern for each specimen was determined by analyzing at least 10 cells. In males, both mitotic (spermatogonial prometaphase) and meiotic (metaphase I or II) plates were observed. For females, only oogonial prometaphases were studied because no meiotic stages can be detected.

The identification of each chromosomal pair was based on size differences and on the analysis of the meiotic configurations. Each pattern can be assigned to the corresponding chromosomal pair only when C-heterochromatin is present in three or four autosomal pairs. To describe the different C-banding patterns, three autosomal morphs, denoted A, B, and C, were recognized on the basis of previous reports (10,12) (Figure 2): A morph (a subterminal C-heterochromatic block is present at one chromosomal end; the other end is euchromatic or has a very small C-band); B morph (C-heterochromatic blocks are clearly present at both chromosomal ends); and C morph (the chromosome is totally euchromatic or has a very small C-band).

We estimated the relative length of the C-heterochromatin in the total length of the autosomal complement. At least three specimens from each population were analyzed. For each specimen, three to five photographs of the gonial metaphase plate were digitized and quantified by means of appropriate software (IPP plus, Media Cybernetics, Carlsbad, CA).

Measuring Genome Size by Flow Cytometry

To establish the haploid genome size, we used flow cytometry to measure nuclear DNA content in gonad cells from 42 male insects (Table 3) previously fixed in ethanol-acetic acid (3:1). Gonads from fixed insects were excised and deposited on excavated glass slides. A few drops of hypotonic DNA-staining buffer (HDSB), containing 0.1% trisodium citrate, 0.1% Triton X-100, 100 µg/mL RNAase A, and 50 µg/mL propidium iodide) were added to cover the tissue. Gonads were then minced by using scalpel blades until homogeneous slurries were obtained. These were transferred with a Pasteur pipette to 5-mL polypropylene tubes, with the glass slides washed with additional HDSB to obtain a final volume of 2 mL. The suspensions

Table l. Analyzed material of *Triatoma infestans* classified by procedence, biogeographic region, altitude, number of specimens analyzed (n) with C-banding, and number of autosomes with C-bands (mean and standard deviation)[a]

Country	Department, province, locality; habitat[b]; y collected	Biogeographic region[c]	Altitude (m)	N (M,F)[d]	No. of C-autosomes mean and SD
Bolivia	La Paz, Murillo, Palomar. D. 1997	Andes [1]	3,000	3 M	18.00 ± 2.00
Bolivia	La Paz, La Paz, Río Abajo. D. 1997	Andes [2]	2,900	16 M	17.00 ± 1.03
Bolivia	Cochabamba, Esteban Arze, Jamach´Uma. D. 1997	Andes [3]	2,700	15 M, 3 F	15.72 ± 1.49
Bolivia	Cochabamba, Esteban Arze, Jamach´Uma. S. 1997	Andes [3]	2,700	7 M, 2 F	16.00 ± 0.87
Bolivia	Chuquisaca, Yamparaez, Uyuní. D. 1997	Andes [4]	2,542	9 M	17.44 ± 0.88
Peru	Arequipa , Arequipa city. D. 1997	Andes [5]	2,336	8 M	16.63 ± 1.06
Bolivia	Cochabamba, Campero, Peña Colorada. D. 1997	Andes [6]	1,890	3 M	16.00 ± 0.00
Bolivia	Santa Cruz, Florida, Pampa Grande. D. 1997	Andes [7]	1,250	4 M	16.75 ± 0.50
Argentina	La Rioja, Anillaco. P. 1997	Austral Chaco [8]	1,400	5 M	6.20 ± 0.45
Brazil	Bahia, Paratinga. D. 1995	Caatinga [9]	500	9 M	6.00 ± 0.00
Brazil	Piaui, Caracol. D. 1996	Caatinga [10]	450	6 M, 8 F	6.00 ± 0.00
Bolivia	Santa Cruz, Cordillera, Izozog. D. 1997	Boreal Chaco [11]	350	2 M	7.00 ± 0.00
Bolivia	Santa Cruz, Cordillera, Izozog. S. “Dark morphs.” 1997	Boreal Chaco [11]	350	8 M	6.00 ± 0.00
Paraguay	Chaco, Río Negro. D. 1997	Boreal Chaco [12]	350	6 M, 3 F	6.33 ± 0.50
Argentine	Córdoba, Cruz del Eje, Los Leones. D & P. 2000	Austral Chaco [13]	250	12 M	5.17 ± 0.58
Argentine	Santiago del Estero, Moreno, San Pablo. P. 1999	Austral Chaco [14]	200	7 M, 3 F	5.50 ± 0.85
Uruguay	Several populations from Southern and Northern. D & P. 1988–1995	Pampeana [15,16]	0–200	44 M, 26 F	5.99 ± 0.12

[a]All specimens came from natural populations. Statistically significant differences ($p < 0.001$) were detected in the number of C-autosomes between Andean (16.54 ± 1.29) and non-Andean (5.93 ± 0.45) grouped samples.
[b]P, peridomiciliary; D, domiciliary; S, sylvatic.
[c]Numbers in brackets refer to the location of the populations in Figure l.
[d]M, males; F, females.

were then incubated for 30 min at 37°C in the dark with occasional vortexing of the tubes. Immediately before flow cytometric analysis, suspensions were filtered through 60-µm nylon mesh. To evaluate absolute DNA contents, we used as reference the Normal DNA Index (Coulter Cytometry, PN 6699500). This reagent consists of normal human lymphocytes fixed in ethanol: acetic acid and is a standard for the human lymphocyte genome size (2 C = 6.436 pg). All measurements were performed on an EPICS XL-MCL flow cytometer (Coulter Electronics, Hialeah, FL) with an air-cooled argon-ion laser tuned at 488 nm and 15 mW. Propidium fluorescence (FL3), proportional to DNA content, was collected through a 650-nm DL dichroic filter plus a 625-nm BP band-pass filter. Forward and side scatter signals were used for morphologic assessment of the samples. Cell aggregates and coincident cells were excluded by analysis of the relationship between FL3 integral and peak signals. DNA content in single cells was determined from FL3 linear histograms. The absolute DNA amount was calculated from the ratio of the mean channel of the insect haploid G0 peak to the mean channel of the human lymphocyte diploid G0 peak. To standardize the measurements, the flow cytometer was calibrated every day with standard FlowSet fluorescent microspheres (Coulter Cytometry), and replicate samples of Normal DNA Index were run with every batch of insect gonad cells.

Results

All *T. infestans* specimens had the same diploid chromosome number (2n = 22), constituted by 20 autosomes and two sex chromosomes (XY in the males and XX in the females). C-heterochromatic blocks were usually located in terminal and subterminal positions. Interstitial C-bands were exceptional. Each specimen exhibited a specific C-banding pattern, without intraindividual variation. Tables 1 and 2 and Figures 1 and 2 summarize the large variability observed in the C-banding karyotype of *T. infestans* from different localities. All populations showed variation in the number and/or the position of C-bands, allowing us to differentiate two clearly distinct groups.

Group 1 includes insects from all Andean populations from Bolivia and Peru (70 specimens). The number of autosomes with C-blocks varied from 14 to 20, with a mean of 16.54 and a standard desviaton (SD) of 1.29 (Figure 2, parts D, E, and F; Table 1). Both sex chromosomes (X and Y) always presented C-bands but with different sizes (Figure 2E). Despite this variation, there was no clear difference in the number of chromosomes with C-bands among populations of the same localities but with different habitats (e.g., sylvatic and domestic populations from Jamach´Uma, Bolivia). Within group 1, the similar size and shape of the 10 chromosomal pairs made it very difficult to identify each pair. The C-heterochromatin content varied from 46% to 56% of the autosomal complement because of the heterochromatin polymorphism already mentioned. The mean haploid DNA content of all Andean specimens (12 insects) measured by flow cytometry was 1.825 ± 0.149 pg (Table 3).

Group 2 includes specimens from all non-Andean populations, whose origins comprise three biogeographic

Table 2. C-banding patterns observed in the three largest autosomal pairs of *Triatoma infestans* from the non-Andean populations analyzed[a]

C-banding pattern	Argentina (Austral Chaco) [8][13][14][b]	Bolivia and Paraguay (Boreal Chaco) [11,12]	Uruguay (Pampeana) [15,16]	Brazil (Caatinga) [9,10]	Total specimens
BB BB BB	-	1	-	-	1
BB BB AB	1	7	6	-	14
BB BB AA	1	3	43	21	68
BB BB AC	1	-	1	-	2
BB AB AA	4	-	16	1	21
BB AA AA	4	1[a]	4	1	10
BB AA AC	3	-	-	-	3
BB AB AC	2	-	-	-	2
BB AB CC	2	-	-	-	2
BB AA CC	1	-	-	-	1
AB BB AA	1	-	-	-	1
AB AB AA	2	-	-	-	2
AB AB AC	3	-	-	-	3
AB AA AB	1	-	-	-	1
AB AA AA	1	3[c]	-	-	4
AA AA AA	-	4[c]	-	-	4
Total	27	19	70	23	139

[a]The population more near the Andean region of Bolivia and Peru, e.g., the Austral Chaco region of Argentine, appeared very variable both in the number of C-banded autosomes and in the karyomorphs observed. By contrast, the samples farthest away from the Andean region, e.g., Brazilian Caatinga populations, were the most homogeneous, almost always exhibiting the same C-karyomorphs (BB BB AA).
[b]Numbers in brackets refer to the location of the populations in Figure l.
[c]Sylvatic (dark morphs).

regions: Chaco (Bolivian and Paraguayan Boreal Chaco, and Austral Chaco of Argentina), Pampeana (Uruguay), and Caatinga (Brazil) (Figure 2A, B, C; Figure 3). The number of autosomes with C-bands varied from four to seven chromosomes (mean 5.93 $\pm$ 0.45) (Table 1), but almost all of the 139 insects presented six C-heterochromatic autosomes (86.33%). In this group, the three first autosomal C-heterochromatic pairs were identified, based on size differences and meiotic configurations. The karyotype described in previous reports, BB BB AA, was by far the most frequent (Table 2 and Figure 3A). The Y chromosome always exhibited C-blocks, whereas the X chromosome did not show any C-banding (Figure 3A). The 30 specimens in this group measured by flow cytometry had a mean of 1.401 $\pm$ 0.111 pg of DNA per haploid nucleus (Table 3) in which the C-heterochromatin ranges from 24% to 30% of the total autosomal length.

Table 1 shows the number of C-heterochromatic autosomes in all samples studied. Table 2 details the C-banding patterns observed within non-Andean populations (group 2). The samples farthest away from the Andean region of Bolivia and Peru, e.g., the Brazilian Caatinga population, were the most homogeneous, almost always exhibiting the same C-karyomorphs (BB BB AA). By contrast, the population from the austral Chaco region of Argentina appeared quite variable, both in the number of C-banded autosomes as well as in the karyomorphs observed (Table 2). In the Andean population, we were unable to identify each chromosomal pair because of the similar size and shape of the autosomes.

Table 3 summarizes the haploid DNA content (expressed in picograms) observed in different populations of *T. infestans*. When group samples were compared, a reduction of 30% from Andean to non-Andean populations was detected. When the Jamach´Uma Domestic sample (Andean Bolivia) was compared with the dark morph population (non–Andean Bolivia) (Table 3), the non-Andean population had 40% less haploid DNA content.

Analysis of Experimental Progeny between Andean and Non-Andean Populations

The meiotic behavior in the hybrids was apparently normal. A complete meiotic pairing was observed between the autosomes, and univalents were not detected (Figure 4). Several asymetric bivalents were clearly observed, formed by one chromosome with C-heterochromatin (one or two C-blocks) and another without C-heterochromatin (Figure 4). We could not detect any alteration in the form of the spermatids and spermatozoids.

Discussion

Chromosomal Groups in *T. infestans*

Our data disclosed two chromosomal groups in *T. infestans* here named Andean (Bolivian and Peruvian Andean samples) and non-Andean (samples from Argentina, Paraguay, Brazil, Uruguay, and Bolivian Chaco). These groups seem discrete and restricted to particular geographic areas; intermediate forms were not detected (Figure 1). These groups may be recognized by using three criteria: 1)

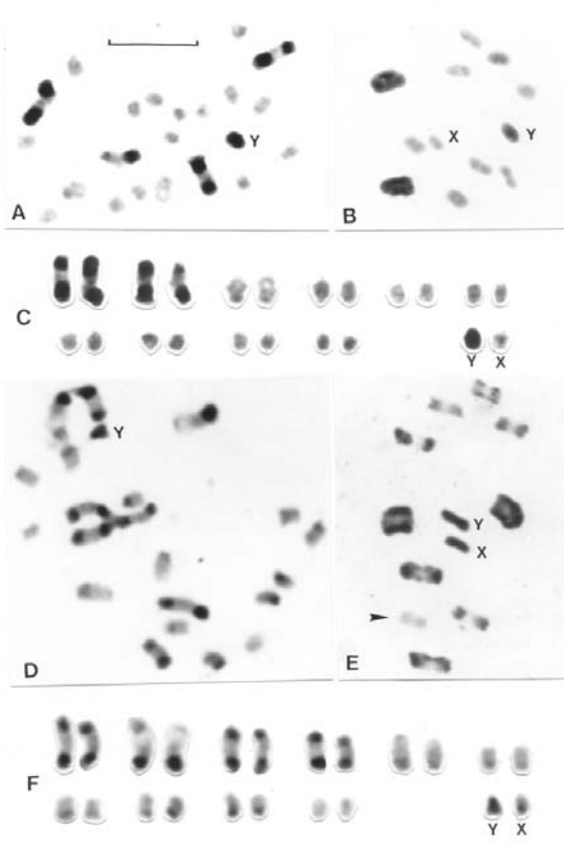

Figure 2. Representative C-banding patterns observed in male *Triatoma infestans*: 2n = 22 (20) autosomes plus XY in males/ XX females) coming from non-Andean (A–C) and Andean regions (D–F). Scale bar = 10 µm. A: Spermatogonial mitotic prometaphase. This specimen from Argentina presents the lowest number of C-banded autosomes (four chromosomes). B: First meiotic metaphase. Only two heterochromatic bivalents, formed by the pairing of the four C-banded autosomes showed in 2A, are observed. The Y (heterochromatic) and X (euchromatic) chromosomes appear as univalents, as typically observed in hemipteran insects. C: Karyotype obtained from 2A. Heterochromatic C-bands are clearly detected in four autosomes and in the Y sex chromosome. D: Mitotic prometaphase in male specimen from Andean Bolivia. Almost all chromosomes present C-bands in one or both chromosomal ends. E: First meiotic metaphase of the same insect shown in 2D. All bivalents except one (arrowhead) are formed by chromosome with C-bands. As observed in other hemipterans, the bivalents form a ring with the univalent sex chromosomes (X and Y) in the center. F: Karyotype obtained from 2D. Chromosome size and C-banding pattern are clearly different from those observed in 2C. Heterochromatic blocks are localized in most autosomes and in both sex chromosomes.

the number of C-banded autosomes, 2) the C-banding on the X sex chromosome, and 3) the DNA content (Figures 2 and 3, Tables 1 and 2). The Andean specimens exhibited consistently more C-banded autosomes (14–20 autosomes) than non-Andean ones (4–7 autosomes); the Andean specimens showed a C-heterochromatic block in the X chromosome, which was absent in the non-Andean specimens, and all of them contained more DNA per cell (approximately 30% more) than did non-Andean specimens (Table 3).

Taxonomic Status of *T. infestans* Populations

Previous studies (8) suggested that heterochromatin could act as a fertility barrier in Triatominae by inhibiting meiotic pairing between chromosomes with different quantities of heteropyknotic regions. However, our analysis of experimental male progeny between both chromosomal groups (F1), where the chromosome pairing takes place without any apparent disturbance (Figure 4), shows that heterochromatin is not a postmating reproductive barrier, at least in *T. infestans*. Moreover, the subsequent developmental cycle and F1 fertility showed no difference with the parental generations (data not shown). Additional evidence for low level of divergence between these populations has been provided by other genetic techniques. Nei's standard genetic distance between Andean and non-Andean populations based on allozyme frequencies was low, generally under 0.050 (7), and the DNA sequence comparison of a 412-bp fragment of the mitochondrial cytochrome B gene showed only three different nucleotide sites (14). At ribosomal DNA level, only 2 transversions and 4 insertions were found among the 459-bp-long ITS-2 (second internal transcribed spacer) between populations from Bolivia (Andean) and Paraguay (Chaco) (15). These data suggest that the genetic variation in the two groups of *T. infestans*, despite their strong chromosomal and DNA content differences, could be attributable to intraspecies variation.

Biologic Significance of Heterochromatin Variation

Eukaryotic genomic DNA contains highly repetitive sequences, the relative amounts of which can differ markedly at population and interspecies levels. Many of the changes in genome size can be attributed to variation in the abundance of these repetitive sequences, rather than to large differences in the nonrepetitive fraction of unique DNA (coding sequences included). C-heterochromatin, revealed by C-banding, consists largely of highly repetitive simple DNA sequences (satellite DNA) and has long been regarded as inert or transcriptionally inactive. However, an extensive literature describes possible adaptive functions and effects of heterochromatin (16). An important and widespread effect of heterochromatin in germ cells both of plants and animals is its influence on the number and distribution of chiasmata. In most organisms, including *T. infestans* (13), the chiasmata either do not form, or form less frequently, in the euchromatic regions adjacent to the heterochromatin segments. Each heterochromatic block, through its chiasma displacement effect, can keep in its proximity certain favorable allele combinations of different genes ("coadapted gene pools") (17). Deletion of C-block

Table 3. Haploid DNA contents (C-value) expressed in pg (mean and standard deviation), measured by flow cytometry, in 42 *Triatoma infestans* specimens from different populations[a]

Origin	Population analyzed[b]	n	Haploid DNA content mean and SD (pg)[c]
Bolivia (Andean)	Jamach'Uma. D. [3]	4	1. 842 ± 0.201
Bolivia (Andean)	Jamach'Uma. S. [3]	4	1. 835 ± 0.140
Bolivia (Andean)	Río Abajo. D. [2]	4	1.799 ± 0.140
Paraguay (non-Andean)	Chaco. D. [12]	4	1.494 ± 0.170
Brazil (non-Andean)	Caracol and Paratinga. D. [9,10]	3	1.420 ± 0.041
Uruguay (non-Andean)	Northern populations. P. D. [16]	13	1.414 ± 0.106
Argentine (non-Andean)	Cruz del Eje and Moreno. P. D. [13,14]	6	1.352 ± 0.094
Bolivia (non-Andean)	Santa Cruz. S. Dark morphs [11]	4	1.320 ± 0.046

[a]n, number of specimens analyzed; P, peridomiciliary; D, domiciliary; S, sylvatic.
[b]Numbers in brackets refer to the location of the populations in Figure l.
[c]Significant differences ($p < 0.001$) were detected in C-values between Andean (1.825 ± 0.149) and non-Andean (1.401 ± 0.111) grouped samples.

can release these zones, allowing recombination to occur and causing certain allele combinations to disappear, generate new ones, or both and as a consequence, influence the adaptability of the individual insect.

Variation in total DNA and heterochromatin contents has also been related to changes in biologic parameters, such as total cell volume, development rate, and body size (18). *T. infestans* specimens from Bolivia are indeed larger than those from Uruguay (7) or Brazil (19), suggesting that heterochromatin amounts could be related to morphologic parameters, and as a consequence, be the target of selective pressures (18).

Origin of *T. infestans*

Based mainly on the existence of sylvatic populations in the Cochabamba valleys of Bolivia, several authors (3,6,20) have suggested that *T. infestans* originated in these Andean valleys. On the other hand, Carcavallo et al. (21) suggested that the origin of this species was in the dry subtropical forest from the South of Bolivia and Paraguay and the North of Argentina. This latter hypothesis was based on the discovery of sylvatic melanic forms of *T. infestans* ("dark morph") in the Bolivian Chaco (22). However, the proposal of the dark morph as the original *T. infestans* population was not supported by body size measurements (23), antennal sensilla patterns (24), or isoenzymatic and mitochondrial data (14). Furthermore, cytogenetic results indicated that in dark morph specimens heterochromatin is restricted to three autosomal pairs (25 and Table 2) and low DNA content (Table 3), suggesting their close relationship with our non-Andean chromosomal group. All these evidences strongly suggest that the dark morphs share a common origin with domestic non-Andean *T. infestans* and that they are not the original population, as suggested by Carcavallo et al. (21).

Domestication Process

Despite some controversy about the origin of *T. infestans*, researchers generally agree that the adaptation of this species to human dwellings began in the Andean regions of Bolivia. There, sylvatic *T. infestans* is found in rock piles associated with small mammals such as wild guinea pigs (*Galea musteloides*) (4). Archaeological findings and historical reconstruction suggest that the domestication process occurred in pre-Colombian times, approximately 3,500 years ago (6), associated with the early settlements

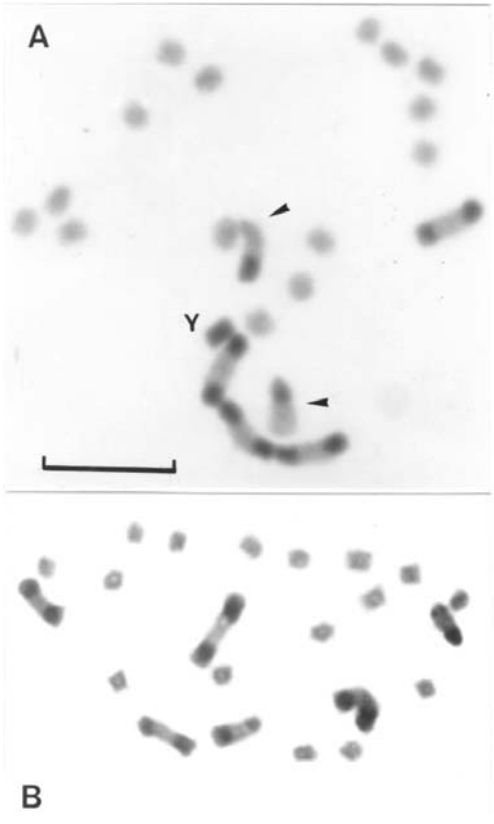

Figure 3. Gonial mitotic prometaphases in male (A) and female (B) specimens of *Triatoma infestans* from non-Andean regions. Scale bar = 10 mm. A: Most common C-banding pattern detected in non-Andean region (BB BB AA). This pattern is constituted by four autosomes with a C-block in both chromosomal ends (B morph) and two chromosomes with a C-block in only one telomere (A morph) indicated by arrowheads. The Y chromosome appears C-heterochromatic. The other 14 autosomes and the X chromosome are C-negative (euchromatic). B: Females only have C-bands in the autosomes; sex chromosomes (XX) are euchromatic and indistinguishable from autosomes without heterochromatin.

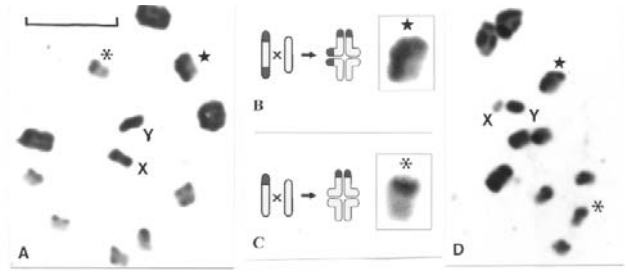

Figure 4. Meiotic pairing in the experimental male hybrid progeny between Andean and non-Andean specimens of *Triatoma infestans*. Scale bar = 10 μm. A: First meiotic metaphase in an insect obtained by crossing a female from Andean region (with C-banded X chromosomes) with a male from non-Andean region. As expected, both sex chromosomes are heterochromatic. As observed in normal specimens, the ten bivalents form a ring with the univalent sex chromosomes in the middle. Chromosome pairing was normal even between chromosomes with great heterochromatic differences. B: Selected bivalent with a diagram of its mitotic and meiotic configuration. As generally observed in hemipteran insects, only a single chiasma is represented. Chromosomes involved in the pairing have different C-patterns: one has C-bands in both chromosomal ends (B-morph); the other one is completely euchromatic (C-morph); the resulting bivalent is asymmetric. C: Selected bivalent with a diagram of its mitotic and meiotic configuration. Chromosomes involved have different C-patterns: one has C-bands in only one chromosomal end (A-morph) while the other one is completely euchromatic (C-morph); the resulting bivalent is also asymmetric. D: First meiotic metaphase in an insect obtained by crossing a female from non-Andean region (with euchromatic X chromosomes) with a male from Andean region. As expected, the X chromosome appears euchromatic in the hybrid. Chromosome pairing was completely normal even between chromosomes with great heterochromatic differences.

of pre-Incaic groups and the domestication of wild rodents for human food. The idea of a discrete Bolivian origin for domestic *T. infestans* is also supported by isoenzymatic studies (7,26). Hence, from Bolivia, domestic *T. infestans* spread over a major portion of South America.

Geographic Spread of *T. infestans* in South America

T. infestans does not fly over long distances and depends mainly on its vertebrate hosts for dispersal; thus, its geographic expansion was most probably associated with human migrations. The settlement of pre-Incaic and Incaic tribes and their spread over substantial Andean regions could be the first series of events allowing passive dispersal of *T. infestans*. However, most of the dispersal of this species appears to have been associated with post-Colombian economic migrations in South America, particularly during the last 100–150 years (6). In Uruguay for example, *T. infestans* appears to have reached some southern communities along the River Plate by 1865 (27), but it was unknown in northern departments of Uruguay until the early 1900s, when it was apparently imported from southern Brazil by human migrations (28). This species also seems to have spread across the Sao Francisco River in Bahia during the early 1970s (29), arriving in the northeastern Brazilian states in the early 1980s (30). This rapid and recent geographic expansion of *T. infestans* from Andean countries to the south of the Neotropical region is supported by its relatively low genetic variability, as measured by isoenzymes (7,26) and mitochondrial (14) and ribosomal DNA sequencing data (15,31).

Origin and Spread of Chromosomal Groups

Andean Dispersal

In light of the historical context mentioned above, *T. infestans* was originally a sylvatic species with large quantities of heterochromatin distributed in most of its chromosomal pairs (autosomes and sex chromosomes). This cytogenetic attribute was not deeply affected during the first phase of its geographic expansion throughout the Andean region of Bolivia and Peru. The domestic specimens in this region constituted an extended population cytogenetically similar to their putative sylvatic original population in central Bolivia (Tables 1 and 3, Figure 2D, E, and F).

Non-Andean Dispersal

This dispersal in non-Andean regions involved *T. infestans* insects with a substantial loss of heterochromatic regions. This reduction is the main cause of the decrease in the DNA size of these insects. Although the mechanisms involved in this heterochromatin loss and DNA size reduction are unknown, several processes have been proposed in other organisms, such as unequal exchange and spontaneous deletion in nonessential DNA (16,32). Non-Andean populations of *T. infestans* could have been established first by one or a few founders that eventually lost part of their heterochromatin by random genetic drift. This kind of founder effect seems to play an important role in the genetic structure of *T. infestans* populations, as has been suggested by isoenzyme analysis (7,26,33). Moreover, the striking similarity among the C-banding patterns found in the non-Andean regions (Table 3), restricted to three heterochromatic pairs, suggests that the event of heterochromatin decrease may have taken place just once in the evolutionary history of *T. infestans*. This finding would imply that current populations of this insect outside Andean regions of Bolivia and Peru all derived from a single group of insects that were restricted to a particular region. Since Austral Chaco *T. infestans* in Argentina have the more variable C-banding patterns of the species from all the non-Andean areas (Table 1) and are geographically close to the Andean region, Austral Chaco was probably the primary focus of dispersal into the non-Andean region. The subsequent dispersion to other regions seems to have

produced populations more homogeneous, in terms of number and localization of heterochromatic regions. Populations of recent colonization, such as those of Brazil and Uruguay, seem to have evolved towards the most common complement with three pairs of C-banded autosomes and a BB BB AA pattern (Figure 3A). In these populations, this karyotype is by far the most frequent and is the only one observed in the most recently colonized zones such as the Piaui state in Brazil (Table 1).

Genomic Changes and Adaptive Processes

Genomic differentiation between both chromosomal groups is likely to be a reflection of both random drift and habitat adaptation. The novel genomic architecture of non-Andean group could have been triggered by a founder event. However, the success of these new small-genome insects is likely associated with adaptation to a new environment. One of the most noticeable differences in the domestic habitats of these groups is the altitude: Andean samples came from geographic regions generally above 1,800 m, whereas non-Andean populations were mainly from localities below 500 m (Table 1). Based on this geographic separation, our working hypothesis is that heterochromatin variation is a reflection of adaptive genomic changes that contribute to the ability of *T. infestans* to survive and reproduce in environments with different altitudes. According to this hypothesis, large-genome populations would be better adapted to Andean (highland) domiciles, while populations with small genomes would do better in non-Andean (lowland) houses. As a consequence, the success and spreading of each chromosomal group into Andean and non-Andean regions may indicate a better adaptation to the different selective pressures of its environment. A positive correlation between chromosome number and heterochromatin content with altitude has been described in other organisms (34,35). Nevertheless, other possible environmental factors or climatic variables associated with Andean and non-Andean habitats should not be discarded.

The inability to detect both chromosomal groups in a same region may also suggest a possible competition between them. The success of one chromosomal group with respect to the other would then depend on altitude. However, large-genome insects would be able to colonize lowlands, and small-genome insects would be able to colonize highlands. This suggestion would explain the colonization by small-genome *T. infestans* of Argentina highlands (as we observed in the Anillaco sample). According to our altitude hypothesis, the Anillaco region should be a primary focus of colonization by *T. infestans* (small genomes), not previously colonized by large-genome insects. The analysis of very close locations with different altitudes in southern Bolivia and northern Argentina would contribute to testing our hypothesis that DNA content reduction reflects adaptive genomic changes related to altitude.

The adaptation of small genome insects to non-Andean domiciles could also be related to a loss in their capacity to return to sylvatic habitats. In non-Andean regions, *T. infestans* does not exhibit sylvatic foci, with the exception of atypical dark morph and melanosoma melanic variants (14,22). These facts could suggest that small-genome insects are unable to adapt to non-Andean sylvatic environments, unless they undergo new genetic changes that influence morphologic parameters.

In summary, we proposed that the genome size decrease observed in *T. infestans* was a successful change as it underwent adaptation to domiciles located in non-Andean lowland regions. However, the founder event generating this genomic variant could have also implied some loss of variability in particular loci. Greater domestic dependence, the inability to return to sylvatic ecotopes, and a certain degree of reduced variability could contribute to making these insects more susceptible to control campaigns, as observed in Uruguay, Chile, and Brazil. In future studies, socioeconomic, environmental, and operational issues also have to be taken into account so that the influence of vector genetic changes in control strategies can be evaluated. Furthermore, the existence of two allopatric groups in *T. infestans* with notable genomic differences is an important feature that have to be considered in evaluating vector control campaigns as well as in selecting the insect used in any genetic studies, including genome sequencing projects.

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Internet Use and Epidemiologic Investigation of Gastroenteritis Outbreak

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In March 2000, a large outbreak of gastroenteritis occurred in a community where a regional computer network provides free Internet access for 42% of the households. We conducted an epidemiologic investigation using the Internet for data collection. Norovirus was identified in stool samples of nine patients but not in the municipal water supply. Of households with access to the network, 19% participated in the survey. The overall attack rate by household was 63%. Drinking water from the nonchlorinated community water system was associated with illness (relative risk [RR] 1.6; 95% confidence interval [CI] 1.1 to 2.2); drinking water only from a private well was associated with decreased likelihood of illness (RR 0.3; 95% CI 0.1 to 0.8). Data collection through the Internet was efficient. Internet surveys may become more common in epidemiologic investigations and have the potential to provide data rapidly, enabling appropriate public health action. However, methods should be developed to increase response rates and minimize bias.

The Internet is increasingly influencing the practice of epidemiology. It has been used for disseminating health information, providing access to journals, and managing multicenter randomized controlled trials (1). During an investigation of a syphilis outbreak (2), the Internet was used to notify patients' sex partners and to increase community awareness. Use of the Internet has also been evaluated as a risk factor for sexually transmitted diseases (3). The Internet provides advantages in data collection and collation, which can reduce the resources and workload required for questionnaire studies (4). However, there are no reports of community-based outbreak investigations conducted by using the Internet.

Noroviruses are common etiologic agents of epidemic gastroenteritis. In March 2000, a widespread norovirus outbreak occurred in a municipality in eastern Finland. We conducted a community-based epidemiologic investigation to determine the magnitude and source of the outbreak. A community computer network and the Internet were used for data collection. We assessed the feasibility and methodologic aspects of Internet-based surveys in an outbreak investigation, including representativeness of the population sample and potential sources of bias.

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Material and Methods

Setting and Description of Computer Network

Ylä-Karjala region comprises municipalities A (population 10,000), B (population 6,700), and C (population 3,000) in North Karelia in eastern Finland. In 1998, an experimental pilot project in the Finnish information society called Learning Ylä-Karjala was begun. The project aims to reduce high unemployment and outmigration by providing a community computer network with free access to the Internet, local discussion groups, local information areas, and email for private citizens, state and municipal authorities, private companies, and other organizations. Approximately 3,500 households (42% of households in the municipalities) with 4,100 persons regularly use the regional network. At the end of 1999, 21% of the residents in municipality A, 20% in municipality B, and 22% in municipality C were registered users of the network. Persons who do not own a computer can access the network from public computers located in libraries, shops, and cafés. Most users (92%), however, access the network from a home computer. Each week, approximately 2,500 registered users log on to the network; 25% of registered users log on daily.

The Outbreak

On March 10, 2000, the National Public Health Institute was notified of an outbreak of gastroenteritis in municipality A. During the next 2 weeks, an increasing

number of patients with vomiting, diarrhea, or both were identified throughout the municipality. The clinical features were typical of norovirus gastroenteritis: 1–2 days' duration and vomiting and nausea as predominant symptoms. On March 24, small spherical viruses suggestive of norovirus were detected in stool specimens of patients by electron microscopy. Wide distribution of cases in municipality A and outbreaks among groups of tourists visiting that municipality who reported drinking municipal tap water suggested that this municipality's water supply may have been contaminated. No increases in incidence of gastroenteritis were reported in neighboring municipalities B and C.

Municipality A's water system provides groundwater to 75% of the population; the rest have private wells. As the water is not routinely chlorinated, the environmental health unit issued a boil-water notice on March 24, and the municipal water works started chlorinating the water. Municipalities B and C have their own, separate water systems.

Epidemiologic Investigation

To investigate the outbreak, we conducted a survey among users of the community computer network in municipalities A, B, and C. Data were collected by using a standard online questionnaire, which is based on FirstClass software (Centrinity Inc., Richmond Hill, Ontario), posted on the network. Only persons who completed the questionnaire from a home computer were included in the study. The questionnaire was placed in a specific message area, and data from the completed questionnaire were transferred directly to a database. Data were transmitted to the server encrypted, and the server was protected by a firewall, both features included in FirstClass software. From April 10 to 24, when a user logged on to the network, a notice appeared on their screen requesting the user to complete the online questionnaire concerning the outbreak of gastroenteritis. To increase the participation rate, the survey was advertised on two different days in the local newspaper.

A case-patient was defined as member of a household with at least one registered user of the community computer network who had an episode of diarrhea (≥3 loose stools/day), vomiting, or both during March 2000. If more than one resident of the household was ill, only the one with first onset of illness was requested to complete the questionnaire. Participants were asked about symptoms, onset of illness, and exposures to drinking water from different sources during March 1 to 31, 2000.

Data were analyzed by using EpiInfo version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA). We calculated attack rates (AR) by municipality and household as well as the relative risks (RR) and 95% confidence intervals (CI) for drinking any water from the municipal water supply, unboiled water from the supply, and well water.

Laboratory and Environmental Investigations

Stool specimens from 23 patients who were treated for acute gastroenteritis at the community health center of municipality A during March 21 to 31 were examined for viruses by electron microscopy and by the reverse transcription–polymerase chain reaction (RT-PCR) test for Norovirus, Sapovirus, and Astrovirus (5,6). Routine bacterial cultures and microscopy for parasites were also performed for the specimens. Nine water samples from the water supply of municipality A were investigated for coliforms and noroviruses, as described previously (7).

Results

Epidemiologic Investigation

A total of 672 persons in the three municipalities completed the online questionnaire. These persons represented an estimated 19% of registered households with access to the network. Of all respondents, 508 (76%) were from municipality A, 59 (9%) from municipality B, and 51 (8%) from municipality C. Forty-one percent of respondents were men, and 59% women. The median age was 27 years (range 6–74). In municipality A, the demographic characteristics of respondents were different from the population of the municipality: the proportion of young adults 15–28 years of age was higher, and the proportion of persons ≥65 years of age was much lower than in the general population (Table 1).

Of respondents, 368 (55%) met the case definition; 60% were female. The median age was 27 years (range 8–70). The epidemic curve (Figure) shows a fluctuating outbreak with several peaks. Cases began to increase in early March; the incidence peaked during March 19 to 23.

Table 1. Attack rates of gastroenteritis by age group in municipality A, eastern Finland, March 2000

Age (y)	Population (%)	Respondents (%)	Cases	AR (%)[a]
0–14	1,743 (17)	72 (14)	43	60
15–28	1,479 (15)	183 (36)	123	67
29–64	4,975 (49)	249 (49)	152	61
≥65	1,911 (19)	4 (1)	1	25
Total	10,108 (100)	508 (100)	319	63

[a]AR, attack rate; based on first episode of illness occurring in the household.

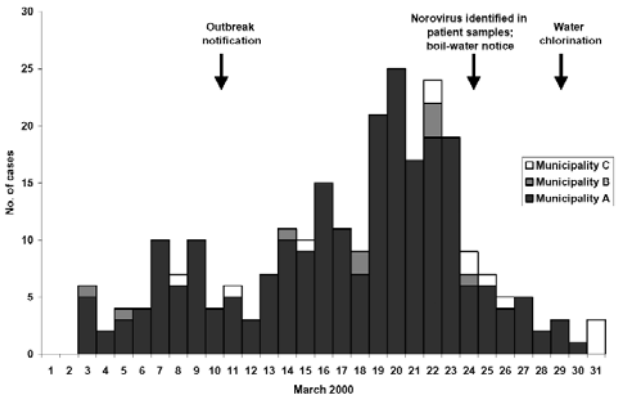

Figure. Cases of gastroenteritis by date of illness onset in a Norovirus outbreak, eastern Finland, March 2000. Based on first episode of illness occurring in the household.

Of the municipalities, the attack rate by household was highest (63%) in municipality A compared with that in municipalities B (17%) and C (29%) (RR 2.8; 95% CI 2.0 to 3.9). By direct standardization of the age-specific attack rates to the population, the total number of ill in municipality A was 5,500. Among residents of that municipality, drinking any unboiled tap water was significantly associated with illness (AR 65% vs. 42%; RR 1.6; 95% CI 1.1 to 2.2), while drinking water from a private well was associated with decreased likelihood for illness (AR 24% vs. 71%; RR 0.3; 95% CI 0.1 to 0.8) (Table 2).

Laboratory and Environmental Investigations

Nine stool specimens (one from a survey participant) were positive for Norovirus by RT-PCR. In two additional patients, electron microscopy results for small, round spherical viruses were positive. All stool samples were negative for bacterial pathogens and parasites. All samples from the municipal water supply were negative for noroviruses by RT-PCR; the samples also were negative for indicator bacteria. Inspection of the water supply system did not indicate the site of contamination.

Discussion

In investigating this widespread Norovirus outbreak, collecting data through the Internet was efficient and yielded a sample of the population with sufficient statistical power. Among residents of municipality A, drinking tap water was associated with illness, whereas drinking well water exclusively was associated with decreased likelihood of illness.

Widespread Norovirus outbreaks are common, and investigations may require substantial resources. In our study, electronic collection of data had two major advantages. First, when questionnaires were completed online instead of through a mail-in or telephone survey, several days were saved. Second, appropriate planning of the electronic questionnaire and direct transfer of data into a database saved much time and allowed a large sample size to be obtained without additional resources. However, Internet data collection may cause other delays. It takes time to set up the Web page and check its proper function. In our study population, only 25% of users logged on daily, and half of registered users did not log on during a whole week, while most people likely answer the phone and check their mail daily.

Our study had several limitations. Of the households in our study area, only 42% had access to the computer network. In telephone surveys, noncoverage is usually not a major concern (8). In community-based Internet surveys, noncoverage may be a substantial problem, depending on the topic studied. Because we only included persons who completed the questionnaire from a home computer, young people were overrepresented among respondents of our online survey. Also, although detailed demographics of registered users of the community network were not available, young age groups and persons with higher education and income are overrepresented among Internet users (1). Only four respondents were ≥65 years, although this group represents nearly 20% of the population. However, representativeness of participants may also be a problem in studies that use traditional methods for enrolling participants such as random digit dialing (9).

In our survey, 19% of households with access participated, but defining the exact sampling frame was not possible. This problem also is similar to telephone surveys using random digit dialing (10). In recent studies using that dialing system, estimated response rates from 28% to 35% have been reported (9,11). In an email study of a defined group of employees in Alaska, 91% of questionnaires were returned (12). Company employees likely have access to a computer and are also accustomed to checking email daily. The higher response rates in email studies are therefore not

Table 2. Factors associated with gastroenteritis among residents of municipality A, Finland[a]

	Exposed			Not exposed				
Risk factor	Ill	Total	AR (%)	Ill	Total	AR (%)	RR	95% CI
Any unboiled tap water	299	460	65	20	48	42	1.6	1.1 to 2.2
Unboiled tap water at home	251	366	69	19	54	35	2.0	1.4 to 2.8
Unboiled tap water outside home	240	347	69	50	112	45	1.6	1.3 to 1.9
Well water only	4	17	24	145	204	71	0.3	0.1 to 0.8

[a]AR, attack rate; RR, relative risk; CI, confidence interval.

directly comparable with our survey. Whether response rates in studies that collect data electronically will ever be as high as in studies that collect information by telephone or mail is not known. In a recent investigation of an outbreak of conjunctivitis at a university, data were collected through email and the Internet, and the response rate was only 50% (13). Telephone or in-person surveys have higher participation rates and fewer missing data than surveys that use self-administered questionnaires (14).

The setting in our study was unusual because a single provider provided access to the Internet, which made it easy to distribute information about the survey to users. Most communities, however, are serviced by several Internet providers, making it more difficult to access users. Whether all commercial providers would be willing to interrupt the log-in procedure with this request and link to the outbreak investigation Web site is uncertain. As was done in our survey, advertising the study in newspapers and other media could be used to increase participation rates. Data access issues could also be problematic if the study were conducted through a commercial Internet provider.

We conducted searches in Medline, ScienceDirect, and ISI Web of Science (Available from: http://isi10.newisiknowledge.com), but we did not find published reports of community-based outbreak investigations in which data were collected through the Internet or through email. Internet surveys are likely to become more common in epidemiologic investigations and have the potential to rapidly provide data to enable appropriate public health action. In industrialized countries, most people will have access to the Internet and email within the next few years, providing epidemiologists increasing opportunities to conduct studies with online data collection. Defining the sampling frame and appropriate design of the questionnaire and database are essential. Response rates and demographics of respondents should be monitored to minimize selection bias. The method of choice for data collection in an outbreak investigation depends on the population and topic studied. Currently, online data collection seems best suited for investigations conducted in well-defined populations with high Internet coverage, where the exposures studied are unlikely to be strongly related to demographic and socioeconomic factors.

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Correlating Epidemiologic Trends with the Genotypes Causing Meningococcal Disease, Maryland

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During the 1990s in Maryland, the incidence of meningococcal infection increased among those 15–24 years of age, in large part because of serogroup C strains. The incidence of serogroup Y infection also increased in some age groups. We analyzed 150 meningococcal isolates by pulsed-field gel electrophoresis (PFGE). Most serogroup Y isolates were classified by PFGE as clonal group 1 or 2, and most serogroup C isolates were in clonal group 3. The proportion of clonal group 2 strains increased from 7.7% in 1992 to 1993 to 52.0% in 1998-1999 ($p < 0.01$). From 1992 to 1999, 96.0% of serogroup C isolates among persons 15-24 years of age were in clonal group 3 compared with 65.6% in persons 0-14 years of age and 64.3% in persons ≥25 years old ($p \leq 0.02$). Similar to epidemic meningococcal infection, the increase in serogroup C meningococcal infection in persons 15–24 years of age was clonal. Overall changes in serogroup Y incidence were associated with a shift in the genotypes of strains causing invasive disease.

Two changes occurred in the epidemiology of meningococcal infection in Maryland during the 1990s. First, meningococcal incidence in persons 15–24 years of age substantially increased and then declined; nearly half of infections in this age group were caused by serogroup C *Neisseria meningitidis* (1,2). From 1990 to 1997, the incidence increased from 0.9 to 2.1 cases per 100,000 in this age group ($p = 0.01$) before declining to baseline in the late 1990s. By the mid-1990s, infection in persons 15–24 years accounted for nearly 30% of all meningococcal infections in Maryland, compared to 16% in 1990 to 1991. This increase was caused mostly by sporadic infections. Only three outbreak clusters were recognized, all caused by serogroup C and representing seven total cases with six cases in persons 15–24 years: two cases in 1995 in children aged 12 and 15 years, who lived on the same street; two cases in 1997, in Maryland college students ages 19 and 21 years, and three cases associated with a party in 1999, in young adults ages 18, 20, and 21 years (3). Although the case-fatality rate in persons 15 to 24 years is generally low, during the 1990s, 2.5% of infections in this age group were fatal. The increase in meningococcal incidence in this age group that was observed in Maryland was also seen in other regions of the United States (2).

Another change in the epidemiology of meningococcal infection that was observed in Maryland during the 1990s was a substantial increase in both the incidence and proportion of infection caused by serogroup Y stains. For example, the incidence of serogroup Y infection more than doubled, from 0.15 to 0.34 cases/100,000 population/year ($p = 0.0009$, Cochran-Armitage test for trend), and the proportion of all meningococcal infections of known serogroup caused by serogroup Y strains steadily increased: 24.6% (15/61) in 1992 to 1993, 29.2% (14/48) in 1994 to 1995, 42.5% (34/80) in 1996 to 1997, and 50.0% (35/70) in 1998 to 1999 ($p = 0.0009$, Cochran-Armitage test for trend). This increase was also statistically significant in both children <15 years of age ($p = 0.002$) and adults ≥25 years (0.007); no increase in serogroup Y infection occurred among persons 15-24 years.

The molecular epidemiology of *N. meningitidis* infection has historically been addressed by using multilocus enzyme electrophoresis (MEE) (4,5). Because of the complicated nomenclature and labor-intensive nature of electrophoretic type (ET) determination, alternative methods have been sought. Recently, multilocus sequence typing (MLST) of housekeeping genes has been shown to highly correlate with ET (6). Pulsed-field gel electrophoresis (PFGE) has also been shown to be a useful method for discriminating between sporadic and outbreak serotype C

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strains (7). In this study, we used PFGE and MLST to determine whether the genotypes of *N. meningitidis*, as identified by PFGE, causing invasive disease correlated with the changes in the epidemiology of infection caused by serogroups C and Y that were observed in Maryland during the 1990s.

Methods

Active, laboratory- and population-based surveillance for invasive meningococcal infection from January 1, 1992, to December 31,1999, that was performed as part of the Maryland Bacterial Invasive Disease Surveillance project (BIDS) was the subject of this analysis (1,8). BIDS is the Active Bacterial Core Surveillance (ABCs) component of the multistate Emerging Infections Program Network coordinated by the Centers for Disease Control and Prevention (CDC) (9). The surveillance case definition was the isolation of *N. meningitidis* from a normally sterile body fluid specimen from a Maryland resident (1).

Meningococcal serogroups were determined by the Maryland Department of Health and Mental Hygiene (DHMH), CDC, and the Norwegian Institute of Public Health using antisera supplied by Difco (Kansas City, MO) and the National Institute for Biological Standards and Control (Potters Bar, UK). The serogroup designation from CDC was used for all but 6 (4%) of 150 isolates. Two isolates were identified as nongroupable by CDC and as serogroup Y by DHMH. Another isolate was identified as serogroup C by CDC and serogroup Y by DHMH. In all three instances, the PFGE pattern was consistent with serogroup Y, and the isolates were therefore classified as such. Two isolates were classified as serogroup C by DHMH but as serogroup B in one case and serogroup Y in another. The PFGE patterns were consistent with those of other serogroup C isolates and therefore these isolates were classified as serogroup C. Finally, one isolate with a PFGE pattern consistent with serogroup Y was classified as serogroup C by both CDC and DHMH. Serogrouping performed at the Norwegian Institute of Public Health with the same subculture as the one used for PFGE identified the isolate as serogroup Y; therefore, this isolate was classified as serogroup Y.

PFGE

PFGE was performed on 74 serogroup C and 76 serogroup Y isolates as previously described (10). Briefly, equal amounts of bacterial suspension and 2% low melting agarose (Sea Plaque, FMC Bioproducts, Rockland, ME) were pipetted into plug molds and incubated in ESP buffer (0.5 M EDTA, 1% N-lauroyl sarcosine, 1 mg/mL Proteinase K; pH 8.5-9.3) overnight at 50°C. After being washed three times with TE (Tris 0.5 M EDTA) buffer at 37°C, the plugs were restricted with 20 U of *Nhe*I (New England Biolabs, Beverly, MA), 330 μg/mL bovine serum albumin, and 200 μl NE Buffer #2 at 37°C overnight. PFGE was performed in a 1% agarose gel by using the following run parameters: 1–30 s for 18 h, 5–9 s for 8 h at 14°C. After the gel was stained with ethidium bromide, the image was digitized on the Bio-Rad Gel Doc 2000 System (Bio-Rad, Hercules, CA). Dendrograms were created with Molecular Analyst/Multi-Analyst programs (Bio-Rad) by using the unweighted pair group method using arithmetic averages (UPGMA), and a position tolerance of 1.5%. The percentage similarity between PFGE patterns was used to assess the relationship between the patterns; PFGE cannot be used to quantify genetic relatedness. The cophenetic correlation, a measure of the correlation between the similarity represented on the dendrograms and the actual Dice coefficient-derived degree of similarity, was calculated for each dendrogram. A minimum 70% correlation is generally considered to be necessary to ensure that the dendrogram accurately represents the similarities between the PFGE patterns. A meningococcal clone was defined as isolates with an indistinguishable PFGE pattern. A PFGE-based clonal group was defined as isolates with a ≤3-band difference (11) or ≥80% similarity on dendrogram or both (7).

MLST

Fourteen serogroup Y and 11 serogroup C strains, selected to represent the range of PFGE patterns identified among our isolates, underwent MLST (Table 1). MLST was performed by using the following seven housekeeping genes (protein products are shown in parentheses): *abcZ* (putative ATP-binding cassette transporter), *adk* (adenylate kinase), *aroE* (shikimate dehydrogenase), *fumC* (fumarate hydratase) *gdh* (glucose-6-phosphate dehydrogenase), *pdhC* (pyruvate dehydrogenase subunit), and *pgm* (phosphoglucomutase) as previously described (6). The ~470-bp fragments were amplified by polymerase chain reaction (PCR) and sequenced with an ABI Prism 377 (PE Applied Biosystems, Foster City, CA) with 5% Long Ranger Gels (FMC Bioproducts, Philadelphia, PA). DNA sequences were determined on both strands. Sequences were assembled with the Auto Assembler DNA Sequence Software Version 2.0 and consensus sequences compared using Sequence Navigator DNA and Protein Sequence Comparison Software (PE Applied Biosystems). Isolates with ≥5 alleles identical to sequence type (ST)-11 or ST-23 were defined as belonging to ST-11 or ST-23 complex, respectively (available from: http://neisseria.org/nm/typing/mlst/ [accessed July 4, 2003]).

Statistical analyses were performed with SAS (Version 8.2; SAS Institute; Cary, NC) and R for Windows version 1.5.1 (available from: http://cran.r-project.org). Exact tests were performed using StatXact (Version 4.0.1, Cytel

Table 1. PFGE and MLST results for selected serogroup C and Y strains

Serogroup Y					
Age group	Culture date	ST	No. alleles in common with ST-23	ST-23 Complex	PFGE interpretation
15–24	3/93	1625	6/7	Yes	Clonal group 1
15–24	2/97	1625	6/7	Yes	Clonal group 1
≥25	8/94	1625	6/7	Yes	Clonal group 1
≥25	7/93	1625	6/7	Yes	Clonal group 1
<15	8/95	1622	6/7	Yes	Clonal group 1
<15	2/97	1622	6/7	Yes	Clonal group 1
≥25	3/97	1622	6/7	Yes	Clonal group 1
<15	5/94	23	7/7	Yes	Clonal group 1
≥25	5/96	23	7/7	Yes	Clonal group 1
≥25	6/96	23	7/7	Yes	Clonal group 2
≥25	11/99	1620	5/7	Yes	Clonal group 2
≥25	10/99	1621	6/7	Yes	Clonal group 2
≥25	10/99	1621	6/7	Yes	Clonal group 2
<15	4/99	167	0/7	No	Neither clonal group 1or 2
Serogroup C					
Age group	Culture date	ST	No. alleles in common with ST-11	ST-11Complex	PFGE interpretation
15-24	3/97	11	7/7	Yes	Clonal group 3; 1997 Outbreak
15-24	2/97	11	7/7	Yes	Clonal group 3; 1997 outbreak
15-24	7/99	11	7/7	Yes	Clonal group 3; 1999 clone
15-24	5/99	11	7/7	Yes	Clonal group 3; 1999 outbreak and clone
<15	8/99	11	7/7	Yes	Clonal group 3
<15	12/96	11	7/7	Yes	Clonal group 3
15-24	4/96	11	7/7	Yes	Clonal group 3
<15	6/93	1626	1/7	No	Not clonal group 3
<15	5/96	1623	0/7	No	Not clonal troup 3
<15	6/97	1060	0/7	No	Not clonal troup 3
≥ 25	6/95	278	0/7	No	Not clonal group 3

[a]ST, sequence type; PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing.

Software Corporation; Cambridge, MA). The Dice coefficients were used to perform the pairwise similarity analysis of the serogroup C isolates by age group. In this analysis, the mean and median Dice coefficients were calculated separately for each age group and period, and the distributions of the Dice coefficients among the groups were compared by using the Kruskal-Wallis rank sum test. For each age group, only one isolate from each of the three small outbreak clusters was included in the temporal analyses; however, all isolates were included in the serogroup distribution, description of serogroup C strains in 1998-1999, and the PFGE dendrogram.

Results

During the study period, 295 cases of meningococcal infection were reported (1), of which 259 (87.8%) were available for serogroup determination. Among these 259 isolates, 98 (37.8%) were serogroup Y, 86 (33.2%) were serogroup C, 49 (18.9%) were serogroup B, 12 (4.6%) were nonserogroupable, 10 (3.9%) were serogroup W-135, 2 (0.8%) were serogroup Z, and 1 (0.4%) was serogroup X.

Seventy-eight percent (76/98) of the serogroup Y and 86.0% (74/86) of the serogroup C strains were available for PFGE analysis. The dendrogram of the 150 strains, which had a cophenetic correlation of 87.3%, is shown in Figure 1. Based on the number of band differences and the percent of similarities on the dendrogram, two clonal groups of serogroup Y (arbitrarily designated as clonal groups 1 and 2) and one clonal group of serogroup C (clonal group 3) were noted. Clonal group 1 consisted of 49 serogroup Y strains, clonal group 2 consisted of 21 serogroup Y strains, and clonal group 3 consisted of 57 serogroup C strains. The remaining six serogroup Y strains were designated "nonclonal 1 or 2 strains," and the remaining 17 serogroup C strains were designated "not clonal group 3" strains.

The proportion of clonal group 2 serogroup Y strains increased from 7.7% (1/13) in 1992 to 1993 to 20.0% (2/10) in 1994-1995,17.9% (5/28) in 1996 to 1997, and 52.0% (13/25) in 1998 to 1999 ($p < 0.01$, exact test for trend). Among the 14 serogroup Y isolates on which MLST was performed (Table 1), clonal group 1 strains

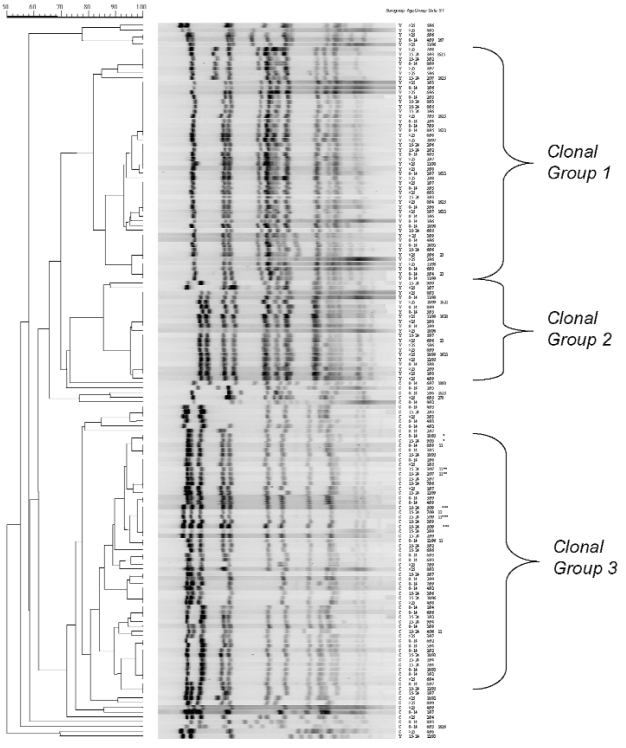

Figure 1. Pulsed-field gel electrophoresis (PFGE) patterns of 150 meningococcal serogroup C and Y strains isolated from persons during 1992–99. Serogroup, age group, culture date, and sequence type (ST) are listed to the right of the dendrogram. The three clonal groups and the PFGE patterns of the two isolates from the 1995 (*) outbreak, two isolates from the 1997 outbreak (**), and three isolates from the 1999 outbreak (***) are indicated. NOTE: For a larger reproduction of this image, please see http://www.cdc.gov/ncidod/EID/vol10no3/02-0611.htm See text for a brief description of outbreaks.

belonged to ST-23, ST-1622, or ST-1625 and clonal group 2 belonged to ST-23, ST-1620, and ST-1621. Overall, the serogroup Y clonal group 1 and 2 strains tested belonged to the ST-23 complex. The nonclonal group serogroup Y strains had STs that were not closely related to the ST-23 complex.

Among the 74 serogroup C strains, 32 (43.2%) occurred among persons 0–14 years of age, 28 (37.8%) occurred among persons ages 15–24 years, and 14 (18.9%) occurred among adults ages ≥25 years. Over the 8-year period, 96.0% (24/25) of persons ages 15–24 years were infected with a serogroup C clonal group 3 strain compared to 65.6% (21/32) of persons ages 0-14 years ($p < 0.01$), and 64.3% (9/14) of adults ages ≥25 years ($p = 0.02$). While the incidence was rising from 1992 to 1997 among persons ages 15–24 years, 94.7% (18/19) of persons in this age group were infected with a clonal group 3 strain compared to 57.7% (15/26) of persons ages 0–14 years ($p < 0.01$) and 60.0% (6/10) of adults ages ≥25 years ($p = 0.04$).

From 1998 to 1999, the period during which the incidence in persons 15–24 years of age had returned to baseline, 87.5% (7/8) of the serogroup C isolates from this age group were due to a single clone within clonal group 3 (Figure 1). This clone was only detected in 1999 among persons ages 15-24 years, including the three party-associated case-patients (3). Strains from the four other 1999 case-patients that had indistinguishable PFGE patterns from the party outbreak strains were neither epidemiologically linked to the outbreak nor occurred in the month before or after the outbreak. For both the 1995 and 1997 outbreaks, the PFGE patterns of the implicated isolates were indistinguishable within the outbreak, yet distinct from the other strains (Figure 1). All clonal group 3 serogroup C strains that underwent MLST, including strains from the 1997 and 1999 outbreaks, belonged to ST-11 (Table 1). The "not clonal group 3" strains had no alleles in common with ST-11.

In the pairwise similarity analysis, serogroup C infection in persons 15-24 years was caused by isolates with more highly-related PFGE patterns than serogroup C isolates from other age groups (Table 2, Figure 2). For example, the median (25th and 75th percentiles) pairwise similarity during 1992 to 1999 for serogroup C isolates that caused infections in persons ages 15–24 years was 87.0% (83.3%; 91.7%), in contrast to 78.3% (63.6%; 87.0%) and 72.7% (64.0%; 81.8%) for persons <15 and ≥25 years of age, respectively ($p < 0.01$ for the comparisons of persons 15–24 years versus each of the other two age groups).

Discussion

From 1992 to 1997, the increasing incidence of invasive meningococcal disease among persons 15-24 years of age was largely caused by a clonal group of serogroup C strains that belonged to the ST-11 complex. These strains had PFGE patterns that were more similar to each other than the serogroup C isolates that were causing infection in the two other age groups. ST-11 (ET-37 complex) has been associated with outbreaks and epidemics (6,7,12). While the meningococcal incidence decreased in this age group in the late 1990s, a unique PFGE-defined serogroup C clone emerged in 1999, which had not been previously detected. Among patients infected with serogroup Y isolates, a shift occurred from one ST-23 complex Y clonal group to another over time.

The risk for meningococcal infection depends on a variety of strain, host, and environmental factors (13). During epidemics, which are generally clonal, the proportion of cases that occur in adolescents and young adults often rises, in addition to an increased incidence in other age groups (14). This pattern is believed to be caused at least in part by the introduction of a new strain to which the

Table 2. Pairwise similarities (% similarity) of PFGE patterns of serogroup C strains, by age group and period

Period	Age group (y)	No. of isolates	25%	50% (Median)	75%	Mean	p value[b]
1992–1999	0–14	32	63.6	78.3	87.0	75.4	<0.01
	15–24	25	83.3	87.0	91.7	86.8	
	≥25	14	64.0	72.7	81.8	74.2	
1992–1997	0–14	26	60.9	76.2	84.6	73.1	<0.01
	15–24	19	80.0	87.0	91.0	85.7	
	≥25	10	61.5	72.7	83.3	73.2	
1998–1999	0–14	6	82.6	87.0	95.7	88.9	<0.01
	15–24	6	91.3	100	100	96.2	
	≥25	4	70.7	72.7	78.2	76.1	

[a]PFGE, pulsed-field gel electrophoresis.
[b]Kruskal-Wallis rank sum test for the comparison of 15–24 year-old group versus each of the two other age groups.

population has little immunity. The increase in adolescents and young adults is similar to the pattern we observed in Maryland, although no associated epidemic occurred. Analogous to the epidemic setting, we hypothesized that the increase in persons ages 15–24 years was due to the introduction of a serogroup C clone. We expected that the increase was caused in large part by this new clone because adolescents and young adults typically otherwise have a low risk for meningococcal infection. This situation contrasts to that of younger children and older adults, who, in general, are more susceptible to meningococcal infection and therefore might be expected to be infected with a broader range of strains (2,15). A similar pattern was recently observed in Oregon, although the increase in that case was caused by a serogroup B clone (14).

Although our study had not begun early enough to determine whether clonal group 3 serogroup C strains were recently introduced into Maryland, the finding that serogroup C infection in persons ages 15–24 years was caused by strains that were more similar by PFGE to each other than serogroup C infection in other age groups generally supports our hypothesis. The emergence of a new PFGE-defined clone in adolescents in 1999 was somewhat surprising since the meningococcal incidence was decreasing in this age group during that time. The shift from one ST-23 complex serogroup Y clonal group to another during the 1990s may also have been associated with changes in population immunity that occur over time because of the circulation of N. meningitidis in the community. However, since we did not study whether the two clonal groups differed in cell surface immunogens, this hypothesis is speculative.

MLST has recently been validated as an alternative method to MEE. We found that PFGE discriminated among strains with the same ET. For example, MLST could not discriminate between the two serogroup C PFGE-defined clones in the 1997 and 1999 outbreaks. This result was similar to the findings of a recent study in which meningococcal isolates with the same ET were found to have distinct PFGE patterns (7). Taken together, these data indicate that PFGE and MLST are complementary methods for studies of the molecular epidemiology of *N. meningitidis* infection (3,7).

In summary, epidemiologic trends in invasive meningococcal disease in Maryland from 1992 to 1999 were associated with specific PFGE-based genotypes of serogroup C and Y strains. Additional studies will be needed to determine whether future changes in meningococcal incidence are associated with variations in the strains causing invasive disease.

Acknowledgments

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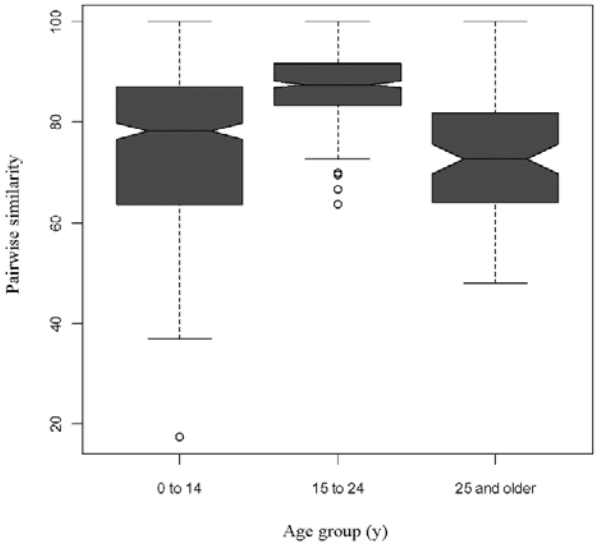

Figure 2. Box-plot of mean pairwise similarities demonstrating the genetic relatedness of serogroup C strains for persons ≤14 years (32 strains), persons 15–24 years (25 strains), and adults, ages ≥25 years (14 strains) during 1992–1999. The lower, central, and upper horizontal lines in the box indicate the 25th, 50th, and 75th percentiles. The outliers, as defined as the 25th or 75th quartile ±1.5x the interquartile range, are plotted as circles. Notches of box plots that do not overlap indicate a statistically significant difference at the <0.05 level.

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I cannot greatly honor minuteness in details, so long as there is no hint to explain the relation between things and thoughts; no ray upon the metaphysics of conchology, of botany, of the arts, to show the relation of the forms of flowers, shells, animals, architecture, to the mind, and build science upon ideas. In a cabinet of natural history, we become sensible of a certain occult recognition and sympathy in regard to the most unwieldy and eccentric form of beast, fish, and insect.

–Emerson

Legionella Infection Risk from Domestic Hot Water

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We investigated *Legionella* and *Pseudomonas* contamination of hot water in a cross-sectional multicentric survey in Italy. Chemical parameters (hardness, free chlorine, and trace elements) were determined. *Legionella* spp. were detected in 33 (22.6%) and *Pseudomonas* spp. in 56 (38.4%) of 146 samples. Some factors associated with *Legionella* contamination were heater type, tank distance and capacity, water plant age, and mineral content. *Pseudomonas* presence was influenced by water source, hardness, free chlorine, and temperature. *Legionella* contamination was associated with a centralized heater, distance from the heater point >10 m, and a water plant >10 years old. Furthermore, zinc levels of <20 mg/L and copper levels of >50 mg/L appeared to be protective against *Legionella* colonization. *Legionella* species and serogroups were differently distributed according to heater type, water temperature, and free chlorine, suggesting that *Legionella* strains may have a different sensibility and resistance to environmental factors and different ecologic niches.

Legionnaires' disease is normally acquired by inhalation or aspiration of legionellae from a contaminated environmental source. The first evidence of the association between potable water from shower and nosocomial legionellosis was reported approximately 20 years ago (1), and the hot water system is thought to be the most frequent source of cases or outbreaks within a hospital (2,3), where patients may be at a higher risk for a severe infection (4–6). Relatively little is known about sporadically occurring cases of community-acquired legionellosis, which accounts for most infections (7,8), although correlation analyses suggest that a substantial proportion of these cases may be residentially acquired and associated with bacteria in hot water distribution systems (9).

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Legionella spp. have been isolated from water with a temperature as high as 63°C, and the contamination is associated with other bacteria and protozoa (10,11). Biofilm formation can provide a means for survival and dissemination of *L. pneumophila* (12,13), interfering with efforts to eradicate bacteria from water systems (14,15). The accumulation of microorganisms on the pipeline surfaces and the formation of biofilms are influenced by many factors, such as surface materials, concentration and quality of nutrients and disinfectants, temperature and hydraulics of the system, and pipe surface roughness (16).

To assess the potential public health impact of *Legionella* colonization at a domestic level, a descriptive multicentric study was undertaken to identify and quantify the levels of the microorganism in a substantial number of Italian domestic hot water samples. *Pseudomonas* spp. are part of the natural population of the water, but some species should be considered as opportunistic pathogens. Furthermore, *Pseudomonas* may compete with *Legionella* to grow in the aquatic environment (17,18); thus we also evaluated *Pseudomonas* colonization.

We addressed three specific aims: 1) to estimate the frequency of *Legionella* colonization and severity of contamination at the domestic level; 2) to identify potential risk factors for contamination relative to distribution systems and water characteristics; 3) and to define the relative role of each risk factor and suggest possible remediation. Lastly, risk for legionellosis was retrospectively evaluated by collecting information about pneumonia symptoms recorded by residents at home.

Methods

Sample Collection

From May through June 2002, a total of 146 water samples were collected from private homes of six towns (Milan, Modena, Bologna, Rome, Naples, Bari) representative of different Italian regions (Northern, Central, and Southern Italy). A similar number of samples were taken from each town; selection was made on the basis of the

water distribution systems inside the town and building and heater types in each area. After we identified each building, we asked a random family (in case of a condominium) to participate in the study, i.e., to complete our questionnaire and give informed consensus for water collection. Laboratory examinations were free, and at the end of the study each participating family received a letter with results of *Legionella* analysis.

Hot water samples were drawn from the bathroom outlets (shower heads or bathroom tap) in three sterile 1-L glass bottles after a brief flow time (to eliminate cold water inside the tap or flexible shower pipe). To neutralize residual free chlorine, sodium thiosulphate was added in sterile bottles for bacteriologic analysis, whereas acid-preserved glass bottles were used for chemical determinations. Collection bottles were returned to the laboratory immediately after sampling for bacteriologic and chemical-physical examination; if analyses would not begin within 24 hours, samples were kept at >4°C and processed within 48 hours of collection.

Microbiologic Analysis

To detect *Legionella* spp., 2-L water samples were concentrated by membrane filtration (0.2-µm-pore–sized polyamide filter, Millipore, Billerica, Massachusetts, USA). The filter membrane was resuspended in 10 mL of original sample water and vortex-mixed for 10 min. To reduce contamination by other microorganisms, 5 mL of this suspension was heat-treated (50°C for 30 min in a water bath) (19). Two aliquots of 0.1 mL of the original and concentrated specimens (heat-treated and untreated, 1:10 diluted and undiluted) were each spread on duplicate plates of modified Wadowsky-Yee selective medium (Oxoid Ltd., Basingstoke, Hampshire, UK). The plates were incubated at 36°C in a humidified environment with at least 2.5% CO_2 for 10 days and read from day 5 at the dissecting microscope. Suspected colonies with a mottled surface or an iridescent and faceted cut-glass appearance, were counted from each sampling. All colonies from plates with ≤10 and 10–20 random colonies were subcultured on buffered charcoal yeast extract (BCYE) agar (with cysteine) and charcoal yeast extract agar (cysteine-free) media (Oxoid) for ≥2 days. Only colonies grown on BCYE were subsequently identified by an agglutination test (*Legionella* Latex Test, Oxoid). The test allows a separate identification of *L. pneumophila* serogroup 1 and serogroups 2–14 and detection of seven *Legionella* (polyvalent) species (other than *L. pneumophila*), which have been implicated in human disease: *L. longbeachae*, *L. bozemanii* 1 and 2, *L. dumoffii*, *L. gormanii*, *L. jordanis*, *L. micdadei*, *L. anisa*. *Legionella*-like bacteria serologically nonidentifiable (in total 4 colonies) are excluded from the account, awaiting a different confirmation by DNA sequence (polymerase chain reaction [PCR]-method). The results are expressed as CFU/L and the detection limit of the procedure was 25 CFU/L (mean value of two plates). All the research units participated in a quality control for *Legionella* detection in water that was organized by the National Health Institute, through a periodic distribution of water samples added with unknown *Legionella* species and concentration. The total microbial counts at 36°C and 22°C were obtained twice by the pour-plate method on plate count agar (Oxoid). The plates were incubated at 36°C for 48 h or at 22°C for 72 h.

To isolate *Pseudomonas* spp., 100-mL and 10-mL water samples were filtered through a 0.45-µm-pore–size membrane (Millipore). If the number of bacteria was high, suitable dilutions were made. The membranes were placed on *Pseudomonas* cetrimide fucidin cephalosporin (CFC) agar (Oxoid) and incubated at 30°C for 48 h. Each type of oxidase-positive colony was counted.

Physical and Chemical Analyses

Water temperature and residual free chlorine (DPD method, colorimetric) were determined at the time of sample collection. Standard techniques were used to measure oxidizability and water hardness. Concentrations of calcium, magnesium, iron, manganese, copper, and zinc were measured by flame atomic absorption spectrophotometer (Perkin-Elmer, Wellesley, MA, mod 5000) on acidified samples (1% HNO_3) concentrated by boiling.

Risk Factors

A detailed standardized questionnaire was developed to evaluate risk factors possibly associated with colonization. The first part collected information on family characteristics (number of components, age and sex, length of stay in residence) and on pneumonia events during their stay in the home. The second part was devoted to home data: type (flat, single house, villa), flats in the building, home floor, home rooms and bathrooms, building age, type of water supply, and disinfection systems used. The third part collected information on the heating system (central or independent, electric or gas heater), distance of the sample site from the water distribution point, existence of a tank and its volume, age of the system, service frequency, and existence and characteristics of a softening and water recycling systems. Water operating temperature (temperature at the distribution site) was also recorded.

Statistical Analysis

All statistical calculations were made with SPSS/pc (SPSS Inc, Chicago, IL). Logarithmic transformations were used in statistical analyses to normalize the nonnormal distributions, and results are presented as geometric means. The bacteriologic data were converted into $\log_{10}$

(x+1). When possible, variables were categorized into dichotomous ones. The results were analyzed by correlation analysis, *t* test, one-way analysis of variance (ANOVA), and by chi-square test. Odds ratios (OR) and 95% confidence intervals (CI) were calculated to assess categorical risk variables associated with microbial contamination. Variables that were significant in the univariate analysis were entered in a multiple logistic regression model. By using conditional logistic regression models, independent predictors of colonization were established. Variables were retained in the model if the likelihood ratio test was significant ($p < 0.05$).

Results

Descriptive Data

Table 1 shows general characteristics of the examined water in terms of supply and distribution systems. Five heating systems were recognized, corresponding to those more frequently used at the domestic level, although with geographic differences, as the centralized systems were mainly adopted in northern Italy.

Table 2 shows chemical and microbiologic qualities of hot water samples. When samples were grouped according to their origin (mixture or groundwater), groundwater had significantly higher levels of calcium (105.7 ± 32.1 mg/L vs. 68.8 ± 31.3 mg/L, $p < 0.001$) and magnesium (21.5 ± 16.0 mg/L vs. 14.9 ± 6.0 mg/L, $p < 0.01$) and was harder (35.4 ± 13.0 vs. 22.1 ± 8.8°F, $p < 0.001$). *Pseudomonas* spp. were isolated from 56 of 146 (38.4%) samples, with levels ranging from 1 to 6.4 x 10^4 CFU/100 mL; 85.7% of positive samples contained fewer than 10^3 CFU/100 mL.

A total of 33 (22.6%) samples of 146 were contaminated by *Legionella* spp., and *L. pneumophila* (Table 3) was the most frequently isolated species (75.8% of isolates). In the positive samples, the mean number of legionellae was 1.17 x 10^3 CFU/L (range 25 to 8.7 x 10^4 CFU/L); three samples (9.2%) contained ≥10^4 CFU/L, none of which were *L. pneumophila* serogroup 1. Although we examined colonies with different morphologic traits, the agglutination test did not reveal multiple species or serotypes in a single water sample.

Univariate Examination of Risk Factors

The risk for microbial contamination according to the system characteristics was evaluated by applying a univariate logistic regression (Table 4). A central warm water system and distance of the water from the heating point >10 m were strongly associated with the risk for *Legionella* contamination (OR 9.24 and 8.10, respectively, $p < 0.001$). Other significant and positive associations were observed with tank volume, plant age, flooring in the home, and total number of flats in the building.

Table 1. Characteristics of water supply and distribution systems in the examined buildings (N = 146)

Characteristic	Frequency no. (%)
Type of water	
Groundwater	67 (46.2)
Mixture water	79 (53.8)
Disinfection	
Cl dioxide	58 (39.7)
Na hypochlorite	30 (20.6)
Cl dioxide + Na hypochlorite	32 (22.2)
None	26 (17.5)
Plumbing material	
Metal	131 (89.7)
Plastic	15 (10.3)
Type of heater	
Independent	
Gas, tank	30 (20.5)
Electric	22 (15.1)
Gas, instant	55 (37.7)
Central	
House	36 (24.7)
Neighborhood	3 (2.1)
Softening system	
Absent	124 (84.9)
Present	22 (15.1)
Hot water circulation	
Absent	117 (80.1)
Present	29 (19.9)
Plant maintenance	
Never/every 2 years	26 (17.8)
Once/year	100 (68.5)
Every 6 months	20 (13.7)

Pseudomonas contamination was positively associated with heating system age and negatively with tank distance and water operating temperature. *Pseudomonas* was also associated with the particular water source, with 65.3% of groundwater colonized versus 12.3% of mixture water (OR 13.44; 95% CI 5.02 to 36.03, $p < 0.001$).

The univariate regression was then applied to study the association between microbiologic data and water chemical parameters (Table 5). Seven factors were independently protective against *Legionella* colonization: high levels of copper, hardness, oxidizability, and free chlorine and low concentrations of zinc, iron, and manganese. Lower levels of zinc and manganese were also associated with lower total count at 36°C and 22°C. *Pseudomonas* was positively associated with total hardness and iron <20 µg/L, whereas residual free chlorine significantly inhibited *Pseudomonas*. When water samples were grouped according to their trace element levels, water samples (n = 12) characterized by concentrations of zinc <100 µg/L, iron <20 µg/L, and copper >50 µg/L were all negative for *Legionella*. No other system or water parameters were associated with bacterial contamination of the examined samples.

Table 2. Chemical, physical, and microbiologic characteristics of the examined hot water samples

Characteristic	Mean	SD	Minimum	Maximum
Ca (mg/L)	85.0	33.0	0.1	252.0
Mg (mg/L)	17.6	10.6	0.1	90.1
Total hardness (°F)	28.3	11.4	0.3	100.1
Sampling temperature (°C)	41.9	12.4	17.0	65.0
Operating temperature (°C)	52.9	10.0	20.0	90.0
	Geometric mean	Median	5th percentile	95th percentile
Fe (µg/L)	15.0	21.0	0.1	171.5
Mn (µg/L)	2.4	3.0	0.1	13.9
Cu (µg/L)	11.5	14.2	0.1	87.5
Zn (µg/L)	62.6	100.0	1	776.4
Free Cl (µg/L)	9.2	26.4	0.0	250.0
Oxidizability (mg/L O_2)	0.63	0.64	0.24	1.70
Pseudomonas spp. (CFU/100 mL)[a]	139.2	223.6	1.0	1.0×10^4
Total count at 36°C (CFU/mL)[a]	98.6	95.0	3.4	1.7×10^3
Total count at 22°C (CFU/mL)[a]	50.4	30.0	4.0	3.9×10^3

[a]Positive samples only.

Multivariate Examination of Risk Factors

The data were reanalyzed by means of multivariate conditional logistic regression models. A central heating system, distance from heating point >10 m, and a system >10 years old were each independently associated with higher risk of *Legionella* colonization, whereas water with levels of copper >50 µg/L and zinc <100 µg/L were predictive of no contamination (Table 6). For *Pseudomonas*, only groundwater remained highly predictive of colonization (OR 12.69; 95% CI 2.66 to 44.00, $p < 0.001$), whereas an operating temperature >50°C was predictive of noncontaminated samples (OR 0.25; 95% CI 0.11 to 0.98, $p < 0.05$).

Risk Factors and Legionella Species

The percentage distribution of *Legionella* species differed significantly according to the heater system (chi-square = 14.00, $p < 0.05$). Electric heaters were legionellae-free; the gas-heated independent systems had little contamination (10.0% of those with tank and 16.4% of those without) and were mainly colonized by either *L. pneumophila* serogroup 1 or non-*pneumophila Legionella* species. In the centralized heating systems of both single buildings and neighborhoods, *Legionella* colonization was higher (52.8% and 66.7%, respectively), and *L. pneumophila* serogroups 2–14 were the most frequently isolated serotypes. Germ concentration did not differ according to the heater type.

Table 7 shows that water temperature, level of free chlorine, and *Pseudomonas* contamination differed according to *Legionella* species (Table 7). Water samples contaminated by *L. pneumophila* serogroup 1 were characterized by significantly lower operating temperature compared to that of the other groups, whereas water samples positive for *L. pneumophila* serogroups 2–14 had lower residual chlorine and higher *Pseudomonas* count.

Risk Assessment

The reported frequency of pneumonia symptoms was double among persons living in the legionellae-positive homes compared to those living in legionellae-free buildings (8 cases in 95 residents vs. 15 cases of 333 residents), but the difference was not significant (OR 1.95; 95% CI 0.80 to 4.75). Results did not change by correcting for the duration of residence of each person in the examined house.

Discussion

In our study, *Legionella* spp. were isolated in 22.6% of domestic hot water samples, with a mean number of

Table 3. Characteristics of *Legionella* contamination in the examined domestic hot water

Characteristic	*Legionella* spp. total	*L. pneumophila* serogroup 1	*L. pneumophila* serogroups 2–14	Other *Legionella* species
Positive samples n (%)	33/146 (22.6)	6/33 (18.2)	19/33 (57.6)	8/33 (24.2)
Count (CFU/L)				
Geometric mean	1.17×10^3	0.96×10^3	0.94×10^3	2.30×10^3
Median	1.85×10^3	0.89×10^3	1.85×10^3	3.16×10^3
5th percentile	54	100	25	100
95th percentile	4.1×10^4	5×10^3	3×10^4	8.8×10^4
Distribution n (%)				
1–9.9×10^2 CFU/L	15/33 (45.4)	3/6 (50.0)	9/19 (47.4)	3/8 (37.5)
10^3–9.9×10^3 CFU/L	15/33 (45.4)	3/6 (50.0)	8/19 (42.1)	4/8 (50.0)
$\geq 10^4$ CFU/L	3/33 (9.2)	0/6 (0.0)	2/19 (10.5)	1/8 (12.5)

Table 4. Univariate analysis of system and building characteristics associated with microbial contamination

Characteristic	OR (95% CI)			
	Legionella spp. yes/no (33/113)	*Pseudomonas* spp. yes/no (56/90)	Total count at 36°C high/low (62/84)[b]	Total count at 22°C high/low (35/111)[b]
Central heater	9.24[c] (3.87 to 22.05)	0.87 (0.40 to 1.85)	0.92 (0.44 to 1.94)	1.36 (0.59 to 3.12)
Distribution site distance >10 m	8.10[c] (3.41 to 19.23)	0.47* (0.22 to 1.00)	1.08 (0.54 to 2.17)	1.51 (0.69 to 3.32)
Tank capacity >100 L	5.21[c] (2.14 to 12.67)	0.58 (0.24 to 1.43)	0.58 (0.24 to 1.39)	0.64 (0.22 to 1.83)
Age of heating plant >10 y	3.24[d] (1.38 to 7.59)	1.85[e] (0.94 to 3.64)	1.14 (0.59 to 2.19)	1.58 (0.73 to 3.43)
House floor >3rd	2.35[e] (1.04 to 5.30)	1.38 (0.66 to 2.87)	0.94 (0.45 to 1.96)	1.24 (0.54 to 2.83)
Apartments >12/building	2.26[e] (0.99 to 5.18)	1.48 (0.75 to 2.91)	1.00 (0.52 to 1.94)	1.13 (0.53 to 2.44)
Sampling temperature >50°C	0.62 (0.26 to 1.49)	0.91 (0.24 to 3.40)	1.16 (0.54 to 2.51)	1.22 (0.54 to 2.75)
Operating temperature >50°C	0.69 (0.29 to 1.67)	0.32[d] (0.15 to 0.70)	1.74 (0.80 to 3.76)	0.57 (0.24 to 1.36)

[a]OR, odds ratio; CI, confidence interval.
[b]High >100 CFU/mL.
[c]$p < 0.001$.
[d]$p < 0.01$.
[e]$p < 0.05$.

legionellae in positive samples of 1.17 x 10^3 CFU/L (geometric mean); the highest concentration was 8.7 x 10^4. In previous studies in Finland and Germany, the occurrence of legionellae was similar (30% and 26%, respectively) as well as the contaminating concentration (20,21). In an Italian study of hot water samples taken from swimming pool showers, 27% were positive for *Legionella* spp. and 46% for *P. aeruginosa* (18), findings in line with results of our study on domestic water plants. According to a survey in Germany (22), *L. pneumophila* is by far the most abundant species in potable and environmental water samples, as >75% of positive samples were contaminated by *L. pneumophila*.

We could not verify seasonal variability in the contamination, because all samples were taken in the spring. Recent studies, however, found that contamination was consistent throughout the year, both in terms of the species of legionellae isolated and in the concentration of organisms (18), suggesting that the occurrence of Legionnaires' disease most frequently in the summer is not necessarily linked to a higher water contamination.

By comparing the environmental factors associated with *Legionella* and *Pseudomonas* occurrence, substantial differences in the microbes' sensitivity to these factors were observed. *Pseudomonas* was not influenced by system characteristics but strongly affected by water parameters. Thus, free chlorine and operating temperature appeared to inhibit these microbes, whereas groundwater origin, which influences higher degree of hardness, was found to favor *Pseudomonas* occurrence. The negative effect of chlorine and the positive influence of hardness, particularly higher calcium level, have been already observed in other studies on *Pseudomonas* water contamination (17,23).

Conversely, system and building characteristics were the main predictors for *Legionella* in domestic hot water. Thus, residing at higher floors of large buildings with many apartments and with older, centralized water heating systems increased the risk for *Legionella* contamination compared to living in apartments with independent water heater systems and a short distance from the sampling point to the hot water distribution site. Among independent heaters, electric ones appeared to be most protective against contamination, whereas the opposite was observed in previous studies in Quebec City, where temperature of electric heaters was significantly lower than that of fossil-fuel

Table 5. Univariate analysis of water chemical parameters associated with microbiologic data[a]

Chemical	*Legionella* spp. (yes/no)	*Pseudomonas* spp. (yes/no)	Total count at 36°C (high/low)[b]	Total count at 22°C (high/low)[b]
	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
Cu ≥50 µg/L	0.14[c] (0.02 to 1.13)	0.99 (0.38 to 2.56)	0.64 (0.24 to 1.68)	0.71 (0.22 to 2.28)
Zn <100 µg/L	0.33[d] (0.14 to 0.76)	1,21 (0.62 to 2.35)	0.38[d] (0.19 to 0.75)	0.29[d] (0.13 to 0.67)
Fe <20 µg/L	0.37[c] (0.16 to 0.85)	1.96[c] (1.00 to 3.86)	050[c] (0.25 to 0.97)	0.74 (0.34 to 1.58)
Mn <3 µg/L	0.42[c] (0.19 to 0.94)	1.50 (0.76 to 2.94)	0.23[e] (0.11 to 0.45)	0.31[d] (0.14 to 0.70)
Hardness >25°F	0.41[c] (0.18 to 0.89)	3.91[e] (1.80 to 8.52)	1.92(0.96 to 3.86)	1.03 (0.47 to 2.26)
Oxidizability >0.5 mg/L O_2	0.42[c] (0.18 to 0.95)	1.12 (0.52 to 2.42)	1.01 (0.49 to 2.07)	0.29[d] (0.12 to 0.68)
Free Cl present	0.51 (0.23 to 1.15)	0.35[d] (0.17 to 0.73)	1.52 (0.73 to 3.13)	0.49 (0.22 to 1.09)

[a]OR, odds ratio; CI, confidence interval.
[b]High >100 CFU/mL.
[c]$p < 0.05$.
[d]$p < 0.01$.
[e]$p < 0.001$.

Table 6. Multiple logistic regression of system and water characteristics associated with *Legionella* contamination[a]

Variable	*Legionella* spp. yes/no OR (95% CI)
Central heater	4.88 (1.61 to 14.76)[b]
Distribution site distance >10 m	4.55 (1.55 to 13.33)[c]
Zn <100 µg/L	0.22 (0.07 to 0.66)[c]
Heating system age >10 yr	3.68 (1.25 to 10.82)[d]
Cu >50 µg/L	0.08 (0.01 to 0.97)[d]

[a]OR, odds ratio; CI, confidence interval.
[b]$p < 0.005$.
[c]$p < 0.01$.
[d]$p < 0.05$.

heaters (24). In a representative sample of Wellington, New Zealand, domestic residences with electrically heated hot water systems, no *Legionella* spp. were isolated by culture, but PCR tests were positive for *Legionella* in 12 homes, some with hot water temperatures >60°C, suggesting that the bacteria are killed during passage through the hot water tank (11).

In our study, *Legionella* presence was not affected by the origin of water (groundwater vs. mixture), pipe materials, water temperature, or concentration of chlorine, and the negative association of *Legionella* with hardness and oxidizability disappeared in the multilogistic regression analysis. When the potable system was adopted, *Legionella* was found in both chlorinated and untreated water, confirming the low efficacy of this disinfecting system on microbe eradication (25). In addition, bacteria from a chlorinated water system may be more resistant to combined and free chlorine than bacteria from unchlorinated systems (26).

The examined domestic water samples were not colonized by multiple serotypes or strains, a common finding in hospitals, hotels, and spas (27–29). This result could depend on different distribution systems and frequency of water use between private and public buildings.

Because the contaminating organism (*L. pneumophila* serogroup 1, *L. pneumophila* serogroups 2–14, or non-*pneumophila Legionella* spp.) was specific to a system, we could examine differences in distribution of species according to the system and water characteristics. These differences have been insufficiently evaluated in previous studies, but recent studies demonstrated that intracellular replication, cytopathogenicity, and infectivity to mammalian and protozoan cells also vary with *Legionella* species (30,31).

Our hypothesis is that *Legionella* strains substantially differ in their sensitivity to environmental risk factors and, as a consequence, may have different ecologic niches. *L. pneumophila* serogroup 1, responsible for approximately 80%–90% of Legionnaires' disease cases (32), was predominantly isolated from independent water heating systems, despite the fact that they were less frequently contaminated. Furthermore, compared with the other legionellae, serogroup 1 was found in water with a lower temperature, less *Pseudomonas* contamination, and a relatively higher residual chlorine concentration. Taking results together, *L. pneumophila* serogroup 1 appears to survive and grow in systems with a short distance between the hot water distribution site and the distal outlets. In agreement with our findings, a recent study on contaminated dental units recovered *L. pneumophila* serogroup 1 in nearly all sites positive for *Legionella* species (33). In these conditions, the possibility of contaminated aerosol inhalation might be more frequent for *L. pneumophila* serogroup 1, despite the fact that this serogroup is not the most frequently isolated in hot water systems. If our hypothesis is correct, most probably simple hygienic procedures, like good cleaning practice and periodically replacing shower heads, would be effective in reducing the number of infections. From our experience with epidemic clusters of nosocomial legionellosis in a hospital mainly contaminated by *L. pneumophila* serogroups 2–14 with rare isolates of *L. pneumophila* serogroup 1, we observed that introducing adequate cleaning procedures in the bathroom and surveillance by health personnel was sufficient to avoid further cases, even when the central hot water distribution systems were not decontaminated (34).

Our findings show the possible effect of trace elements on *Legionella* in hot water samples. Experimental studies have shown that *Legionella* spp. are affected by osmolarity (35) and metal concentration (36) and that iron limitation in vitro reduces bacteria growth and expression of the zinc-metalloprotease that is an important pathogenicity

Table 7. Differences in some water parameters according to *Legionella* species

Characteristic	*L. pneumophila* serogroup 1 (n = 6) Mean ± SD	*L. pneumophila* serogroups 2–14 (n = 19) Mean ± SD	*Legionella* other species (n = 8) Mean ± SD	F score (p)
Sampling temperature (°C)	29.4 ± 11.8	48.9 ± 4.4[a]	31.2 ± 15.5	15.17 (<0.001)
Operating temperature (°C)	43.3 ± 12.1[a]	53.7 ± 5.9	57.0 ± 4.4	6.91 (<0.005)
	Geometric mean	Geometric mean	Geometric mean	
Free chlorine (µg/L)	78.0	2.6[a]	32.3	7.86 (<0.002)
Pseudomonas spp. (CFU/100 mL)	0.4	19.1[b]	0.6	3.31 (<0.05)

[a]$p < 0.05$ versus the other two groups.
[b]$p < 0.05$ versus *Legionella* other species.

factor (37). We show that hot water samples low in iron, zinc, and manganese, but rich in copper, predicted the absence of *Legionella* colonization, confirming their roles as growth promoters or inhibitors.

Of particular interest is the inverse relationship between copper levels and *Legionella* presence. In the examined water, the risk of *Legionella* contamination was approximately six times lower when copper levels exceeded 50 μg/L, without influencing *Pseudomonas* contamination. In other studies, copper concentrations low enough to be commonly found in drinking water reduced numbers of coliform bacteria (13). Thus, we emphasize that this trace element influences some, but not all, bacterial growth (33). To control *Legionella* in hot water systems, methods that release copper and silver ions electrolytically in water may represent a promising solution (38–41). Although both metals play a role in limiting bacterial colonization, copper seems to better penetrate biofilm. Amoebae, the natural hosts of legionellae, have not been controlled successfully in vitro by adding metal (42), suggesting that legionellae survive inside protozoa and are destroyed by metal ions when released into free water.

The risk of getting pneumonia was 1.95 higher among residents in the legionellae-positive homes than in residents of the legionellae-negative buildings, but the difference was not significant and was similar to that found in previous studies (19). Legionellae concentrations of 3–7,000 CFU/L could be sufficient to produce one case per year in a susceptible population (43), and these contamination levels correspond to those found in our study at the domestic level. In a recent epidemiologic survey on seropositivity in residents of homes with and without *Legionella* in the water systems, the prevalence of anti-*Legionella* antibodies was twice as high in persons in homes with legionellae as in those persons whose homes did not have legionellae (44). The antibodies were most likely the result of asymptomatic infections caused by exposure in their home water supply, as no cases of pneumonia in the exposed population were reported. Most cases of sporadic legionellosis are not reported to health authorities in Italy as well as in other countries, and finding an association with a specific source of infection such as domestic contamination is rare (45).

Our observations suggest that *Legionella* species should be considered when examining environmental contamination, which is essential to better evaluate environmental risk factors and select the most appropriate prevention and control measures (46). To limit *Legionella* colonization at the domestic level, we suggest simple and general measures: 1) use independent domestic water heaters, 2) maintain high cleaning standards, 3) periodically replace components of the system which could favor presence or dissemination of bacteria, and 4) have a water copper content >50 μg/L. We do not believe disinfecting measures at the domestic level are needed, considering that our retrospective study on pneumonia in residents did not show a relevant evidence of risk in colonized buildings.

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Cysticercosis-related Deaths, California

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Cysticercosis is an increasingly important disease in the United States, but information on the occurrence of related deaths is limited. We examined data from California death certificates for the 12-year period 1989–2000. A total of 124 cysticercosis deaths were identified, representing a crude 12-year death rate of 3.9 per million population (95% confidence interval [CI] 3.2 to 4.6). Eighty-two (66%) of the case-patients were male; 42 (34%) were female. The median age at death was 34.5 years (range 7–81 years). Most patients (107, 86.3%) were foreign-born, and 90 (72.6%) had emigrated from Mexico. Seventeen (13.7%) deaths occurred in U.S.-born residents. Cysticercosis death rates were higher in Latino residents of California (13.0/10^6) than in other racial/ethnic groups (0.4/10^6), in males (5.2/10^6) than in females (2.7/10^6), and in persons >14 years of age (5.0/10^6). Cysticercosis is a preventable cause of premature death, particularly among young Latino persons in California and may be a more common cause of death in the United States than previously recognized.

Cysticercosis, an infection caused by the larval form of the pork tapeworm, *Taenia solium*, is recognized as an increasingly important cause of severe neurologic disease in the United States (1–3). In the typical transmission cycle, eggs from the adult tapeworm are shed in the feces of a human carrier and subsequently ingested by pigs, the usual intermediate host (4). Larvae emerge from the eggs, penetrate the intestinal mucosa and disseminate through the bloodstream to various tissues where the larval stage or cysticercus develops. The cycle is completed when humans, the only naturally infected definitive host, consume raw or undercooked pork containing cysticerci, which attach to the small bowel and develop into adult tapeworms, thereby completing the cycle. However, humans may also become infected with the larval stage when eggs, which are directly infectious, are ingested, typically through contaminated food or water. Neurocysticercosis, the most severe form of the disease, occurs when larvae invade tissue of the central nervous system. While cysticercosis in the United States principally affects immigrants from cysticercosis-endemic areas of Latin America, it has been increasingly recognized in U.S.-born residents as well (5–7).

Despite the growing importance of cysticercosis, surveillance systems for cysticercosis have rarely been implemented (6,8), and the true impact of the disease in the United States is largely unknown. Although several case series have been published (1,2,9–11), these have been facility-based efforts and, consequently, may not provide an accurate measure of either the incidence or severity of the disease. Such facility-based reports have suggested that deaths from cysticercosis in the United States are uncommon. Although population-based data would provide a more accurate assessment of cysticercosis death rates, such data are scarce. To measure cysticercosis-related death rates in California, we reviewed state mortality records for the 12-year period 1989–2000.

Methods

Data Source

Data on deaths were obtained from the State of California, Center for Health Statistics, Office of Vital Records. Completion of a death certificate is required by state law. All death certificates in California require the assignment of a cause or sequence of events leading to death as determined by the attending physician. If a physician is not in attendance, or the death is accidental or occurs under suspicious circumstances, cause of death is determined by the local coroner or medical examiner. Completed death certificates are transmitted from county jurisdictions to the California Department of Health Services, where the causes or sequence of events for each death record are keyed into a computer to create an input data file, which is subsequently sent to the National Center for Health Statistics (NCHS), which produces codes for both the underlying cause of death and multiple cause for each death record. The resulting coded file is then returned to the California Department of Health Services, Office of

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Vital Records. Data from the State of California's Multiple Cause of Death Files for the 12-year period 1989–2000 were searched for persons for whom cysticercosis (ICD-9 code 123.1 for years 1989–1998, and ICD-10 code B69 for years 1999 and 2000) was listed as a cause of death. Additional information extracted from the death record included age, gender, race/ethnicity, level of education, country of birth, place of death, and date of death.

Data Analysis

Cysticercosis death rates per million population were calculated for California and for its major counties by using mid-period population estimates (1994). Population data were obtained from the Department of Finance, Demographic Research Unit, State of California. Crude cysticercosis death rates and 95% confidence intervals were computed according to age, gender, race/ethnicity, and county of residence. Rate ratios and 95% confidence intervals were also calculated. The chi-square, Fisher exact, and Student *t* tests were employed when appropriate to assess apparent differences.

Results

A total of 124 cysticercosis deaths (mean 10.3 per year) were identified over the 12-year study period, representing a crude 12-year death rate of 3.9 per million population (95% confidence interval [CI] 3.2 to 4.6). Latino residents accounted for 115 (92.7%) of the total deaths recorded, while 5 (4.0%) were white, 3 (2.4%) were Asian, and 1 (0.8%) was black (Table 1). Eighty-two (66.1%) were male; 42 (33.9%) were female. The mean age at death was 39.9 years (range 7–81 years). Most case-patients (107, 86.3%) were born outside the United States, and 90 (72.6%) had emigrated from Mexico. All three fatal cases in Asians were in male immigrants >55 years of age. Cysticercosis was listed as the underlying cause of death for 92 (74.2%) of the patients.

Crude cysticercosis death rates are presented in Table 2. Rates were highest in Latino persons ($13.0/10^6$, 95% CI 10.6 to 15.3) compared with those for other racial/ethnic groups ($0.4/10^6$, 95% CI 0.1 to 0.7), in males ($5.2/10^6$, 95% CI 4.0 to 5.7) relative to females ($2.7/10^6$, 95% CI 1.9 to 3.5), and in persons >14 years of age ($5.0/10^6$, 95% CI 4.2 to 5.9). Cysticercosis deaths varied by year with the greatest number (16 deaths) observed in 1992 (Figure). The highest cysticercosis death rates were in the counties of Los Angeles ($7.7/10^6$), Riverside ($6.0/10^6$), and Ventura ($5.7/10^6$). More than half of the deaths (70) were among Los Angeles County residents.

Seventeen cysticercosis deaths occurred in U.S.-born residents, representing 14% of all cysticercosis deaths. U.S.-born patients with fatal cysticercosis had higher educational levels ($p = 0.02$), were older (mean age 49.1 years versus 38.4 years, $p < 0.05$), and were more likely to be male (but this difference was not statistically significant) (Table 3). Although 71% of U.S.–born deaths occurred among Latino persons, this figure was lower than the proportion for foreign-born Latino residents ($p < 0.01$).

Table 1. Demographic characteristics of 124 patients with fatal cysticercosis in California, 1989–2000

Characteristic	No.	%
Sex		
Male	82	66.1
Female	42	33.9
Race/Ethnicity		
White	5	4.0
Black	1	0.8
Latino	115	92.7
Asian/Pacific Islander	3	2.4
Age group (y)		
5–14	3	2.4
15–24	25	20.2
25–34	34	27.4
35–44	16	12.9
45–54	20	16.1
55–64	10	8.1
≥65	16	12.9
Educational level (y)		
<12	83	66.9
12	24	19.4
>12	17	13.7
Country of birth		
United States	17	13.7
Mexico	90	72.6
Other	17	13.7

Principal coexisting conditions listed as contributing to death included hydrocephalus in 45 (36.3%) persons and epilepsy or convulsions in 20 (16.1%) deaths. Reported place of death included inpatient facility (69%), emergency room or outpatient clinic (10%), nursing home, (9%) and residence (9%).

Discussion

Our findings indicate that cysticercosis is an important and preventable cause of premature death in California, particularly among Latino youth, and the disease may be a more common cause of death in the United States than previously recognized. Although fatal cysticercosis principally affects Hispanic immigrants, our findings suggest that this larval tapeworm causes infection and death in U.S.-born residents as well.

Published studies from large facility-based case series have reported that the cysticercosis death rate is relatively low (Table 4). In a review of records from four county hospitals in Los Angeles County, Richards and colleagues identified 497 cysticercosis cases from 1973 to 1983 (1). The overwhelming majority of patients were of Latino eth-

Table 2. Crude cysticercosis death rates by gender, race/ethnicity, and age group and respective rate ratios, California, 1989–2000

	No.	Rate/10^6 (95% CI)[a]	RR[b] (95% CI)
Sex			
Male	82	5.2 (4.0 to 5.7)	1.9 (1.3 to 2.8)
Female	42	2.7 (1.9 to 3.5)	Referent
Race/Ethnicity			
White	5	0.3 (0.04 to 0.5)	Referent
Black	1	0.5 (0 to 1.3)	1.7 (0.2 to 14.3)
Latino	115	13.0 (10.6 to 15.3)	43.3 (17.7 to 106.1)
Asian/Pacific Islander	3	0.9 (0 to 2.0)	3.0 (0.7 to 12.6)
Age group (y)			
5–14	3	0.6 (0 to 1.4)	Referent
15–24	25	5.8 (3.5 to 8.1)	9.7 (2.9 to 32.0)
25–34	34	6.1 (4.1 to 8.2)	10.2 (3.2 to 33.1)
35–44	16	3.1 (1.6, 4.6)	5.2 (1.5 to 17.7)
45–54	20	5.7 (3.2 to 8.3)	9.5 (2.8 to 32.0)
55–64	10	4.4 (1.7 to 7.1)	7.3 (2.0 to 26.6)
≥65	16	4.7 (2.4 to 7.1)	7.8 (2.3 to 26.9)

[a]12-year rate calculated by using midpoint (1994) population data.
[b]Rate ratio.

nicity (95%). The observed death rate was 2.2% (11 case-patients). Another study found three (1.3%) fatal cases of cysticercosis among 238 cases identified in a Los Angeles hospital from 1981 to 1986 (9). All cases were in Latino patients. A third Los Angeles report involving 230 patients observed two deaths (<1%) in a population composed predominantly of immigrants from Latin America (10). A review of neurocysticercosis records from Ben Taub Hospital in Houston, Texas, over the 6-year period 1986–1991 identified 112 cases; 97% were in Latino patients, who were principally from Mexico (2). Deaths were uncommon; one patient died. He also had ventricular disease with shunt malfunction complicated by staphylococcal and candidal infections. Forty-seven cases of neurocystcercosis were reported among children whose cases were diagnosed at Children's Memorial Hospital in Chicago from 1986 to 1994 (12). A total of 45 (96%) patients were Latino; however, 19 (42%) were reportedly born in the United States. No deaths were observed in these pediatric case-patients. In a study from Children's Hospital in Los Angeles covering the period 1980–1986, no deaths occurred among 52 children with neurocysticercosis (11). A total of 51 (98.1%) patients were Latino, 29 (57%) of whom were born in the United States. An unweighted estimate of death rates across these studies suggests a case-fatality rate of <1%. However, such facility-based studies, while providing valuable information, have substantial limitations and may have underestimated the impact of cysticercosis as a cause of death. Limited data from a surveillance system established in Los Angeles County from 1988 to 1990 showed a mortality rate of nearly 6% (8 of 138 incident cases); however, this observation was based on small numbers (6).

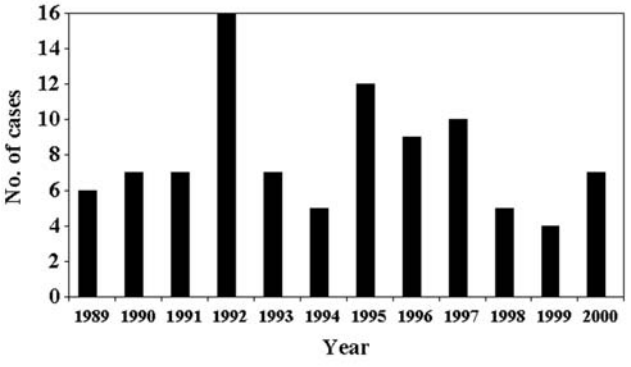

Figure. Cysticercosis deaths in California by year, 1989–2000, from state mortality data.

Table 3. Comparison of selected characteristics of patients born in the United States and those born outside the United States with fatal cysticercosis, California, 1989–2000

	U.S.-born[a] no (%)	Non–U.S.-born[a] no. (%)
Sex		
Male	13 (76.5)	69 (64.5)
Female	4 (23.5)	38 (35.5)
Race/Ethnicity		
White	4 (23.5)	1 (0.9)
Latino	12 (70.6)	103 (96.3)
Black	1 (5.9)	0
Asian/Pacific Islander	0	3 (2.8)
Age group		
5–14	0	3 (2.4)
15–24	3 (17.7)	22 (20.6)
25–34	2 (11.8)	32 (29.9)
35–44	3 (17.7)	13 (12.2)
45–54	1 (5.9)	19 (17.8
55–64	2 (11.8)	8 (7.5)
>64	6 (35.3)	10 (9.3)
Educational level (y)		
<12	7 (41.2)	76 (71.0)
12	5 (29.4)	19 (17.8)
>12	5 (29.4)	12 (11.2)

[a]N = 17.

Table 4. Hospital-based cysticercosis case series and observed mortality rates, United States

Location	No. cases	No. deaths (%)	Y	Reference
Los Angeles	497	11 (2.2)	1973–1983	1
Los Angeles	238	3 (1.3)	1981–1986	9
Los Angeles	230	2 (0.9)	1981[a]	10
Houston	112	1 (0.9)	1986–1991	2
Chicago	47	0	1986–1994	12
Los Angeles	52	0	1980–1986	11

[a]Range of years not provided.

Both the location of the infection and the number of larvae may have an impact on whether the disease is fatal. Extraparenchymal infection, particularly intraventricular disease with resultant hydrocephalus, has been associated with a poorer prognosis (13–15). We found that more than one third of patients who died had concurrent hydrocephalus, suggesting intraventricular location of cysts. In addition, ingesting a large number of eggs may cause an overwhelming, fatal acute infection with numerous larvae and notable central nervous system pathology. Cases of racemose cysticercosis, a phenomenon in which cysticerci continue to grow and spread through tissue, may also have a poor prognosis (16).

Our data suggest that, although uncommon, cysticercosis-related deaths routinely occur among persons born in the United States, a phenomenon that has not been previously reported. Such deaths in U.S.-born residents may reflect low-level endemic transmission within the United States. Confirmed cysticercosis among persons who have never traveled outside the United States has been repeatedly documented over the past 20 years (1,5,6) and can often be traced to a tapeworm carrier among household members or other close personal contacts. Cysticercosis acquired in the United States may also have been transmitted through consuming imported or local produce contaminated with *T. solium* eggs. *Taenia* eggs can survive for long periods in the environment (17), and human feces used as fertilizer or contaminated water employed for irrigation can contaminate crops prior to importation.

Alternatively, cysticercosis among U.S.-born persons may reflect travel-related exposure and infection. Travel by U.S. residents to cysticercosis-endemic areas is common, and exposure to food and water contaminated with the eggs of *T. solium* may readily occur. The recovery of *Taenia* spp. eggs from several varieties of vegetables obtained in local markets in the northeastern Mexican state of Tamaulipas, which borders the United States, has been reported (18). Although probable travel-associated cysticercosis has previously been documented (6), neither the frequency nor the risk factors for travel-related cysticercosis have been studied.

Nearly one half of those who died of cysticercosis in our study were 15–34 years of age. This represents a heavy toll among persons in young, highly productive age groups. Such a phenomenon is uncommon for most infectious diseases, which typically cause higher rates of death in the very young and the elderly (19).

Observed death rates were highest in Los Angeles, Riverside, and Ventura counties. This finding may reflect the fact that residents of these jurisdictions include substantial numbers of immigrants from cysticercosis-endemic areas, particularly Mexico and other areas of Latin America.

Our data, although population-based, likely underestimate cysticercosis deaths for several reasons. Cysticercosis must be recognized and diagnosed for it to be listed on the death certificate. This would require confirmation of infection through biopsy, autopsy or specialized serologic testing (20). Consequently, some cases of fatal cysticercosis likely are undiagnosed and unrecognized; this would result in the miscoding of cysticercosis-related deaths as other conditions. For this reason, death records likely possess moderate sensitivity for identification of true cysticercosis deaths. Our findings demonstrate the benefits of using multiple cause of death data instead of the traditional underlying cause of death data alone for estimating the extent of cysticercosis deaths. An additional 32 (26%) cases were identified by using multiple-cause coded files.

Using death certificates to assess the impact of disease has both advantages and limitations. Since submitting a death certificate is required by state law, virtually all deaths are ascertained and registered. The use of death records, therefore, provides population-based data that avoid the potential biases of facility-based or other non-population-based data sources. Death records also provide a good measure of disease trends over time and can be useful in making comparisons between geographic regions. Mortality data can also help indicate disease severity and contribute to measures of disease impact. Limitations of mortality data include the following: inaccurate information due to errors in recording cause of death; coding errors; and misclassification of race/ethnicity (21–23). Recognizing that deaths from cysticercosis represent only a small fraction of total disease is also important. Finally, both census data and intercensus population estimates used to calculate rates may contain inaccuracies. For these reasons, our estimate of the cysticercosis death rate must be interpreted with caution.

Cysticercosis is a preventable fecal-oral transmitted infection that can cause severe neurologic disease and death and result in substantial cost to the healthcare system. Additional information is needed on the prevalence and incidence of cysticercosis and on cysticercosis-related deaths in the United States. To better define the extent of cysticercosis, state and local health authorities should consider instituting a requirement for the mandatory reporting of this infection. Such surveillance systems should include aggressive efforts to identify possible tapeworm carriers among household members and other close personal contacts. Treating such tapeworm carriers can eliminate sources of infection and prevent additional transmission. Controlled epidemiologic studies to assess risk factors and potential sources for both local and travel-associated cases of cysticercosis should be pursued. Although we could not assess whether problems in access to health care contributed to cysticercosis deaths, nearly 20% of persons with fatal cases died at home, in an emergency room, or in an outpatient setting. Studies to evaluate the possible impact of access issues on cysticercosis deaths would be useful. Collaborative studies with Mexican public health authorities on the prevalence and incidence of cysticercosis in the border regions should be implemented (24). While transmission of cysticercosis from a commercial food handler has never been documented such transmission may occur and any food handler with taeniasis (infection with adult *T. solium* or *Taenia* of unknown species) should be precluded from handling food until successfully treated.

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Amoebae-resisting Bacteria Isolated from Human Nasal Swabs by Amoebal Coculture

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Amoebae feed on bacteria, and few bacteria can resist their microbicidal ability. Amoebal coculture could therefore be used to selectively grow these amoebae-resisting bacteria (ARB), which may be human pathogens. To isolate new ARB, we performed amoebal coculture from 444 nasal samples. We recovered 7 (1.6%) ARB from 444 nasal swabs, including 4 new species provisionally named *Candidatus Roseomonas massiliae*, *C. Rhizobium massiliae*, *C. Chryseobacterium massiliae*, and *C. Amoebinatus massiliae*. The remaining isolates were closely related to *Methylobacterium extorquens*, *Bosea vestrii*, and *Achromobacter xylosoxidans*. Thus, amoebal coculture allows the recovery of new bacterial species from heavily contaminated samples and might be a valuable approach for the recovery of as-yet unrecognized emerging pathogens from clinical specimens.

Free-living amoebae have been isolated worldwide from soil, water, air (1), and from the noses of healthy persons (2,3). Most amoebae feed on bacteria, and few bacteria can resist digestion by the amoebae after being internalized. Amoebal coculture could therefore be used to selectively grow these amoebae-resisting bacteria (ARB) and clean the samples of other more rapidly growing species that generally overwhelm the agar plates. ARB include established agents of pneumonia such as *Legionella* spp. (4) and *Chlamydophila pneumoniae* (5), and potential human pathogens, such as *Parachlamydia* spp. (6,7) and *Legionella*-like amoebal pathogens (LLAPs) (8).

Human exposure to *Parachlamydia* spp. has been especially well demonstrated. Indeed, two of the eight *Parachlamydiaceae* strains isolated to date were found within amoebae recovered from the nasal mucosa (3). However, the prevalence of this potential human pathogen, of other ARB, and of their amoebal host on the nasal mucosa remains to be determined. The fact that *Parachlamydia* is endosymbiotic at 30°C and lytic at 37°C suggests that the bacteria present within amoebae in the cooler upper respiratory tract may be liberated when the amoebae reach the warmer lower respiratory tract (9). This simultaneous liberation of bacteria may overwhelm the human immune response and explain the role of *Parachlamydia* as an agent of community-acquired pneumonia (6) and of aspiration pneumonia (10).

We hypothesized that, for any ARB, nasal colonization may be a first step towards lower respiratory tract infection. Moreover, ARB may use free-living amoebae as a training ground for the selection of virulence traits, as demonstrated for *Cryptococcus neoformans* (11). Thus, virulent capsular strains were phagocytosed by and replicated in *Acanthamoeba castellanii*, leading to amoebal death, while the nonvirulent acapsular strains were killed (11). The fact that free-living amoebae may promote the expression of virulence traits was also well demonstrated for *L. pneumophila* (12,13) and *Mycobacterium avium* (14). A growing body of evidence support the hypothesis that free-living amoebae play a role in the adaptation of *L. pneumophila* to life within other phagolysosomal cells, such as human macrophages (15–18). Moreover, the growth of *M. avium* in amoebae, which results in enhanced entry into both amoebae and macrophages (14), further supports the hypothesis that adaptation to life within human macrophages may be acquired after exposure to environmental predators such as free-living amoebae. Thus, free-living amoebae may be considered as an evolutionary crib for potential emerging human pathogens.

We intended to isolate new ARB from nasal swabs and to compare the prevalence of free-living amoebae and of ARB, such as *Parachlamydia acanthamoebae*, in healthy participants, homeless persons, and hospitalized patients. We tried to recover ARB and free-living amoebae by using amoebal coculture and amoebal enrichment, respectively, with any recovered amoebae subsequently examined for the presence of ARB.

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Methods

Patients and Study Design

Nasal swabs were taken from 100 patients hospitalized in the infectious diseases unit of the Hôpital Nord of Marseille, France, and from 244 homeless persons. In addition, nasal swabs were taken from 100 healthy participants. All participants took part freely in the study and gave their written informed consent. Amoebal coculture was performed for all 444 samples, while amoebal enrichment on nonnutrient agar was restricted to the samples taken from the 100 healthy volunteers and the 100 hospitalized patients.

Broth and Media

Broth and media were slightly modified from the method of T.J. Rowbotham (19). Peptone yeast-extract glucose (PYG): 100 g proteose peptone (Difco, Sparks, MD), 10 g yeast-extract (Merck, Darmstadt, Germany), 4.9 g $MgSO_4.7H_2O$, 5 g sodium citrate.$2H_2O$, 0.1 g $Fe(NH_4)_2(SO_4)_2.6H_2O$, 1.7 g KH_2PO_4, 1.97 g $Na_2HPO_4.7H_20$, 45 g glucose, and 0.295 g $CaCl_2$ in 5 L of distilled water. Page's modified Neff's amoebae saline (PAS): 120 mg NaCl, 4 mg $MgSO_4.7H_2O$, 4 mg $CaCl_2.2H_2O$, 142 mg Na_2HPO_4, and 136 mg KH_2PO_4 in 1 L of distilled water. Nonnutritive agar plates: 1.5 g agar (Research Organics, Cleveland, OH) was diluted in 100 mL of PAS. All media were autoclaved 15 min at 121°C, and broths were also filtered through a 0.22-µm pore membrane (Corning, New York, NY).

Amoebal Microplates

An *Acanthamoeba polyphaga* strain, Linc-AP1, was grown at 28°C in 150-cm^2 cell culture flasks (Corning) with 30 mL PYG. After 5 days, the amoebae were harvested and centrifuged at 179 x *g* (1,000 rpm) for 10 minutes. The supernatant was removed, and the amoebal pellet was resuspended in 50 mL of PAS. Centrifugation and resuspension steps were repeated twice. After the last centrifugation, the amoebae were resuspended in PAS, and 1 mL of $5x10^5$ *A. polyphaga*/mL was distributed in each well of a 12-well Costar microplate (Corning).

Nasal Samples

Nasal swabs were vortexed for 30 seconds in 1 mL of PAS in individual sterile tubes. The suspension was centrifuged at 179 x *g* (1,000 rpm) for 10 minutes. Two hundred and fifty microliters of supernatant was spread onto amoebal microplates. The pellet was spread on nonnutritive agar plates previously flooded with a turbid suspension of viable *Enterobacter aerogenes* in PAS, drained, and incubated at 28°C for a few hours.

Amoebal Coculture

The microplates were centrifuged at 2,879 x *g* (4,000 rpm) for 30 minutes and incubated at 32°C in a humidified CO_2 atmosphere. The amoebal cocultures were subcultured on fresh amoebae on days 4 and 7 (samples from controls and patients) or on day 6 (samples from homeless persons). Amoebal cocultures were examined regularly for amoebal lysis. When amoebal lysis was observed and at the time of subculture, the cultures were screened for intra-amoebal bacteria. This screening was achieved by gently shaking the microplates to suspend the amoebae. Then 150 µL of the suspension was cytocentrifuged at 150 x *g* (800 rpm) for 10 minutes, and slides were stained with Gimenez (20). Fuchsin-stained bacteria easily could be seen within the malachite green-stained amoebae. When Gimenez-stained bacteria were observed, the culture was subcultured on BCYE agar (BioMérieux, Marcy L'Etoile, France) and sheep blood agar (BioMérieux) and on amoebal microplates without antibiotics or with vancomycin (10 µg/mL) or colistin (500 U/mL).

Amoebal Enrichment

Nonnutritive agar plates not supplemented with amphotericin B were incubated at 28°C in a humidified atmosphere and examined daily for amoebae-like cells. When positive, these amoebae-like cells were subcultured on nonnutritive agar plates seeded with viable or UV-inactivated *Enterobacter aerogenes*. After several subcultures on inactivated *E. aerogenes*, recovered amoebae were tentatively grown in axenic PYG broth with or without antimicrobials.

Phenotypic Characterization

Microscopic properties of the isolated bacteria were studied by Gram and Gimenez staining. To confirm that the isolated strains were the Gimenez-stained bacteria, 5 x 10^5 *A. polyphaga*/mL suspensions were injected with the isolated strain and incubated at 32°C. After 4 days' incubation, culture broth was cytocentrifuged and stained with Gimenez and Gram, as described above.

Genotypic and Phylogenetic Characterization

DNA was extracted according to the manufacturer's instructions by using Chelex Resin (BioRad, Hercules, CA) or FastDNA kit (Bio101, Carlsbad, CA) and FastPrep120 grinder (Bio101), depending on the time of strain recovery. Polymerase chain reaction (PCR) amplification of the 16S rDNA gene was performed by using the fD1 and rP2 primers (21) and Taq DNA polymerase (GibcoBRL, Life technologies, Rockville, MD), according to manufacturer instructions. The success of the amplification was determined by electrophoresis in 1% agarose gel of PCR products stained with ethidium bromide. PCR

products were purified by using the QIAquick PCR purification kit (Qiagen, Courtaboeuf, France). Sequencing was performed with the dRhodamine Terminator Cycle sequencing Ready Reaction with one of nine different primers and with AmpliTaq DNA (Perkin-Elmer Biosystems, Warrington, England), according to the manufacturer's instructions. Sequences were determined on a 3100 ABI Prism automated sequencer (Applied Biosystems, Courtaboeuf, France), according to the manufacturer's instructions. Sequences derived from each primer were aligned, compared, and combined in a single 16S rDNA sequence, using the Autoassembler software version 2.1 (Applied Biosystems). The validity of the sequence obtained was assessed by comparison with two additional sequences obtained similarly but from two other PCRs of the same template DNA. The sequences were compared with sequences available in the GenBank database in October 2002 by using the BLAST 2.2.2 program available on the NCBI Web site (www.ncbi.nlm.nih.gov) (22). When the closest sequence was that of an as-yet unpublished bacterial species, the 16S rDNA sequence homology of the prototype strain of the closest published bacterial species was also identified (Table). The 16S rDNA sequences of the isolates were aligned with the sequences of the best blast hits and with those of the closest relative type strains of each isolate. Sequences were edited by removal of the longer 5′ and 3′ ends so that their lengths matched that of the shortest sequence. The 16S rDNA homology of the edited sequences was then analyzed by the distance matrix program of the MEGA software (32). We considered 16S rDNA sequence similarity to be that of the closest sequence in GenBank of more than 99% and 97% for identification at the species and genus level, respectively (33). With these sequences, neighbor-joining (Kimura 2-parameter), minimum evolution (Kimura 2-parameter), and parsimony (standard parsimony) trees were constructed with the MEGA software (32).

Electron Microscopy

For some strains, reinoculation procedure was also performed onto $5x10^5$ *A. polyphaga*/mL suspension in 50-cm^3 flasks (Corning). Four days' coculture was harvested, washed in monophosphate buffer (pH 7.2), and fixed in 4% glutaraldehyde. Fixed samples were washed overnight in monophosphate buffer (pH 7.2), and then fixed for 1 hour at 4°C with 1% osmium tetroxyde. Dehydration was performed by successive washes in increasing acetone concentrations (25%–100%). After incubating the preparations for 1 hour in a vol/vol suspension of acetone/epon and then overnight in epon, they were embedded in Araldite (Fluka, St Quentin Fallavier, France). Thin sections were cut from embedded blocks with an Ultracut microtome (Reichert-Leica, Marseille, France), deposited on copper grids coated with formvar (Sigma-Aldrich, Taufkirchen, Germany), and stained 10 minutes with a solution of methanol-uranyl acetate and lead nitrate with sodium citrate in water. Grids were examined with a Morgagni 268D electron microscope (Philips, Eindhoven, the Netherlands).

Table. Species and family names of 7 Gimenez-positive, gram-negative bacteria isolated from human noses by amoebal coculture[a]

Nb.	Taxonomy of the isolated strain[b]	16S rDNA homology of closest GenBank sequence	16S rDNA homology of the closest published species	Species name	Reference
	Alphaproteobacteria				
	Methylobacteriaceae				
1	*Candidatus Roseomonas massiliae* (AF531769)	99.9% (AF538712)	98.8% (AF533352)	*Roseomonas gilardii*	(23)
2	*Methylobacterium extorquens* (AF531770)	100% (AF293375)	100% (AF293375)	*M. extorquens*	(24–26)
	Bradyrhizobiaceae				
3	*Bosea* sp. strain 7F (AF531764)	99.2% (AF288308)	99.2% (AF288308)	*Bosea vestrii*	(27)
	Rhizobiaceae				
4	*C. Rhizobium massiliae* (AF531767)	100% (AJ389908)	98.7% (Z30542)	*R. larrymoorei*	(28,29)
	Betaproteobacteria				
	Alcaligenaceae				
5	*Achromobacter xylosoxidans* (AF531768)	99.5% (AF394171)	99.5% (AF411021)	*Achromobacter xylosoxidans*	(30)
	Bacteroidetes				
	Flavobacteriaceae				
6	*C. Amoebinatus massiliae* (AF 531765)	99.2% (AB035150)	95.3 % (AJ271009)	*Chryseobacterium scophtalmum*	(31)
7	*C. Chryseobacterium massiliae* (AF531766)	98.0% (AY043370)	96.3 % (AJ271009)	*Chryseobacterium scophtalmum*	(31)

[a]The 16S rDNA homology of the closest GenBank sequence (BLAST analysis) and that of the closest published species with its name are also included. GenBank accession no. are shown in parenthesis. Nb., isolate number.
[b]Order and family are given.

Statistical Analysis

The mean number of strains isolated from ill hospitalized patients was compared to the mean number of strains isolated from the other participants (healthy participants and homeless cohort) by using the student *t* test (STATA software version 7.0., Stata Corporation, College Station, TX).

Results

Amoebal Enrichment

We observed trophozoites growing on nonnutrient agar injected with 1 of 200 samples but failed in propagating them due to fungal contamination, despite subcultures on nonnutrient agar not supplemented and supplemented with amphotericin B (2 µg/mL). Thus, no free-living amoebae could be successfully recovered. Fungal contamination was frequent, occurring in 61% of cultures from patients and 41% of cultures from control samples. These fungi were not identified.

Amoebal Coculture

By amoebal coculture, we recovered seven (1.6%) strains of ARB from nasal swabs of 4 of 444 participants. Five isolates were recovered from 100 patients hospitalized in the infectious diseases unit of the Hôpital Nord of Marseilles, whereas only two isolates were recovered from the 344 other participants (244 homeless and 100 healthy volunteers; p = 0.017). All ARB were gram-negative bacilli. No amoebae-resisting fungi were detected. On the basis of 16S rDNA sequence analysis, the isolated ARB were assigned to alphaproteobacteria (n = 4), betaproteobacteria (n = 1), and the Bacteroides-Cytophaga-Flexibacter group (n = 2). The Table shows the taxonomy and the 16S rDNA homology with GenBank sequences of the seven isolated ARB. When we used a 16S rDNA sequence similarity of more than 99% and 97% for identification at the species and genus level, respectively (33), four (57%) ARB belonged to new species (isolates 1, 4, 6, and 7), including one that also corresponded to a new genus (isolate 6).

Isolate 1, *Candidatus Roseomonas massiliae*, was recovered from a 44-year-old healthy homeless man. The 16S rDNA sequence of isolate 1 shared on BLAST analysis 99.9% homology with a *Roseomonas* sp. implicated in a catheter-related bacteremia (X.Y. Han, unpub. data) and 98.8% with *R. gilardii* strain ATCC 49956^T (Table). Genetic analysis confirmed that isolate 1 belonged to the genus *Roseomonas*, as it shares 93.9%–98.9% 16S rDNA sequence homology with *Roseomonas* spp. and 94.3%–84.6% with other *Methylobacteriaceae*, respectively. Isolate 1 clustered with *Roseomonas* spp. by phylogenetic analysis of the 16S rDNA gene (Figure 1). Thus, bootstrap values of 98%, 99%, and 90% in neighbor-joining, minimum evolution, and parsimony analyses, respectively, supported the fork separating *Roseomonas* spp. (including isolate 1) from their closest relatives, *Craurococcus roseus* and *Paracraurococcus ruber*. More importantly, the isolate was clearly different from the type strain neighbor (*R. gilardii*), with bootstrap values of 100% in neighbor-joining, minimum evolution, and parsimony trees. Isolate 1 has been deposited in the Collection de l'Institut Pasteur, Paris, France, as *Candidatus Roseomonas massiliae* strain CIP 107751^T.

Isolate 2 was recovered from a 27-year-old homeless man with chronic cough, headache, and polylymphadenopathy. The 16S rDNA sequence of isolate 2 shared on BLAST analysis 99.3% homology with *Methylobacterium extorquens* strain JCM 2802^T (Table). Genetic analysis confirmed that isolate 2 is an additional strain of *M. extorquens* as it shared 99.3% 16S rDNA sequence homology with *M. extorquens* strain JCM 2802^T and 94.6%–99% with the other *Methylobacterium* spp. Moreover, isolate 2 clustered with *Methylobacterium* spp. by phylogenetic analysis of the 16S rDNA gene (Figure 1).

Isolate 3 was recovered from a 48-year-old HIV-positive man with cerebral toxoplasmosis and severe pneumo-

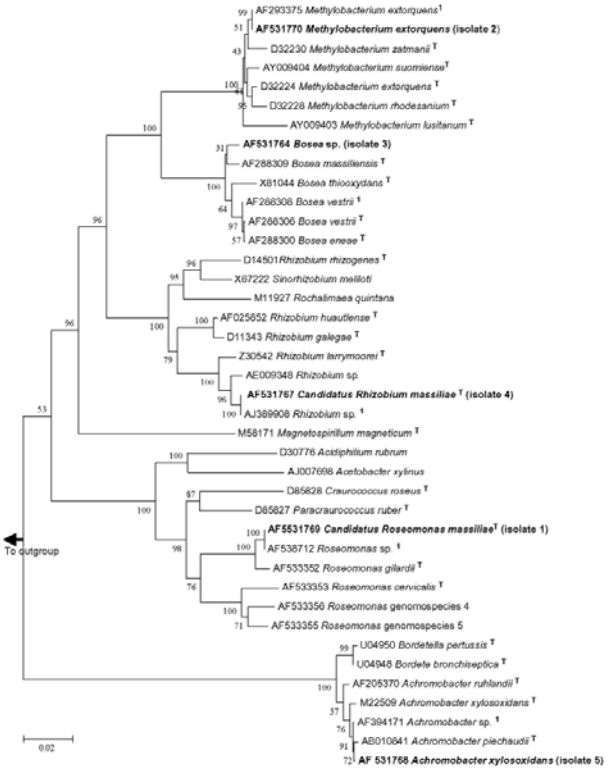

Figure 1. 16S rDNA tree showing relationship of isolates 1 to 5 with related alpha- and beta-proteobacteria. The tree was constructed by using the neighbor-joining method, based on the nearly complete sequence (1,289 nt) of the 16S rDNA gene. Bootstrap values resulting from 100 replications are at branch points. *Staphylococcus aureus* was used as an outgroup.

nia attributed to *Streptococcus pneumoniae*. The 16S rDNA sequence of isolate 3 shared on BLAST analysis 99.2% homology with *Bosea vestrii* strain 34635^T (Table). Genetic analysis confirmed that isolate 3 belonged to the genus *Bosea*, as it shares 98%–99% 16S rDNA sequence homology with *Bosea* spp. Isolate 3 clustered with *Bosea* spp. by phylogenetic analysis of the 16S rDNA gene (Figure 1). However, it clustered with *B. massiliensis*, with bootstrap values separating them (isolate 3 and *B. massiliensis*) from the other *Bosea* spp. of 100%, 56%, and 100% in neighbor-joining, minimum evolution, and parsimony analyses, respectively. The morphology of isolate 3 within *A. polyphaga*, as seen by electron microscopy, is shown in Figure 2.

Isolate 4, *Candidatus Rhizobium massiliae*, was recovered from an 80-year-old woman with pacemaker-associated endocarditis. The 16S rDNA sequence of isolate 4 shared on BLAST analysis 98.7% homology with *Rhizobium larrymoorei* strain 3–10 (Table). Although the isolate shared greater homology with sequences annotated as *Rhizobium* sp., *Agrobacterium* sp., or *A. tumefaciens* (*A. tumefaciens* was renamed *R. radiobacter* [34]), it shared 92.4% sequence homology with *R. radiobacter* strain IFO 13257^T (accession no. D14501). Isolate 4 clustered with two as yet unclassified strains of *Rhizobium* sp. by phylogenetic analysis of the 16S rDNA gene with a node separating them from the closest type strain neighbor, *R. larrymoorei* supported by bootstrap values of 100%, 50%, and 100% in neighbor-joining, minimum evolution and parsimony trees, respectively. Isolate 4 has been deposited in the Collection de l'Institut Pasteur, Paris, France, as *Candidatus Rhizobium massiliae* strain CIP 107749^T.

Isolate 5 (like isolates 4 and 7) was recovered from an 80-year-old woman with a pacemaker-associated endocarditis. The 16S rDNA sequence of isolate 5 shared on BLAST analysis 99.5% homology with *Achromobacter xylosoxidans* strain ATCC 9220^T (Table). Genetic analysis confirmed that isolate 5 belonged to the genus *Achromobacter*, as it shares a 98.4%–99.5% 16S rDNA sequence homology with *Achromobacter* spp. and 97.6%–97.9% with *Bordetella* spp. Isolate 5 clustered with *Achromobacter* spp. by phylogenetic analysis of the 16S rDNA gene (Figure 1). Thus, bootstrap values of 100% in neighbor-joining, minimum evolution, and parsimony trees supported the fork's separating the *Achromobacter* spp. (including isolate 5) from *Bordetella* spp.

Isolate 6, *Candidatus Amoebinatus massiliae*, (like isolate 3) was recovered from a 48-year-old HIV-positive man with cerebral toxoplasmosis and severe pneumonia attributed to *S. pneumoniae*. The 16S rDNA sequence of isolate 6 shared on BLAST analysis 99.2% homology with that of a pathogenic strain belonging to an unpublished new genus and 95.3% with *Chryseobacterium scophtalmum* strain

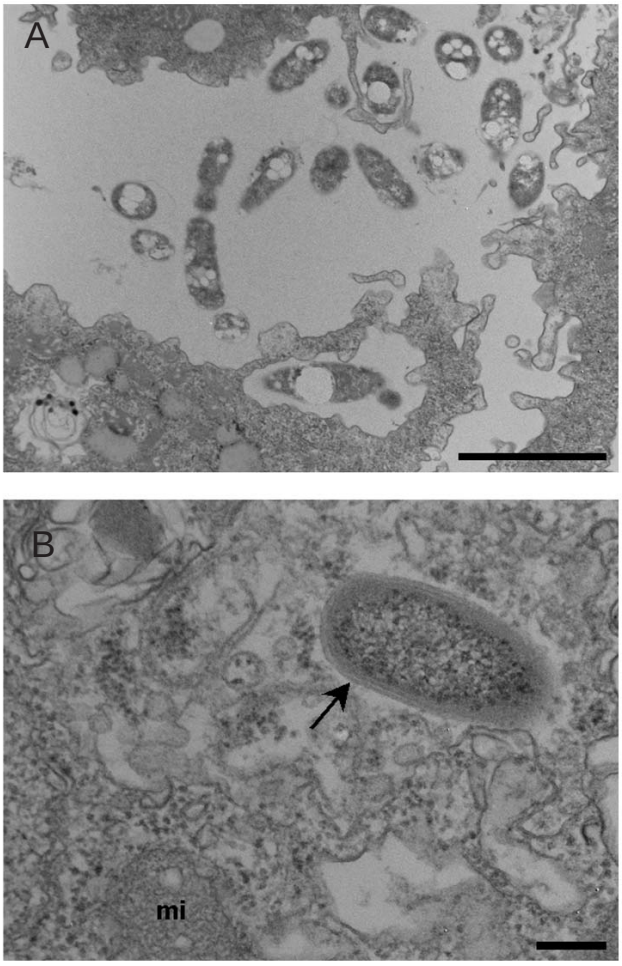

Figure 2A. *Bosea* sp. (isolate 3) in the process of being phagocytized by *Acanthamoeba polyphaga* and in the extracellular media, as seen on electron microscopy. 8,900x magnification. Bar represents 200 nm. B. *Chryseobacterium*-like rod (isolate 7) within *A. polyphaga* (arrows), as seen on electron microscopy. Arrow show the trilammelar membrane. mi = amoebal mitochondria. 36,000 x magnification. Bar represents 200 nm.

LMG 13028^T (Table). On genetic analysis, isolate 6 shared 93.1%–96.0% 16S rDNA sequence homology with *Chryseobacterium* spp. and 81.5%–90.0% with other *Flavobacteriaceae*, respectively. Phylogenetic analysis of the 16S rDNA gene also suggested that isolate 6 belonged to a new genus, related to the *Chryseobacterium* spp. (Figure 3). Thus, bootstrap values of 100%, 100%, and 34% in neighbor-joining, minimum evolution, and parsimony analyses, respectively, supported the fork's separating both *Chryseobacterium*-like bacteria (including isolate 6) from their closest relatives. Isolate 6 has been deposited in the Collection de l'Institut Pasteur, Paris, France, as *Candidatus Amoebinatus massiliae* strain CIP 107750^T.

Isolate 7, *Candidatus Chryseobacterium massiliae*, (like isolates 4 and 5) was recovered from an 80-year-old woman

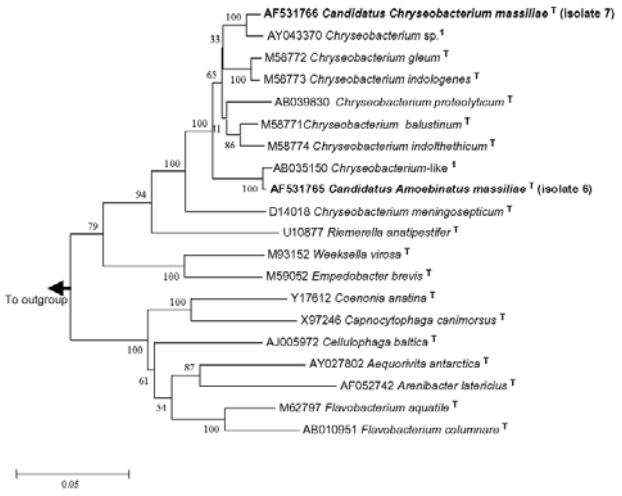

Figure 3. 16S rDNA tree showing relationship of isolates 6 and 7 with related *Flavobacteriaceae*. The tree was constructed by using the neighbor-joining method, based on the nearly complete sequence (1,283 nt) of the 16S rDNA gene. Bootstrap values resulting from 100 replications are at branch points. *Staphylococcus aureus* was used as an outgroup.

with pacemaker-associated endocarditis. The 16S rDNA sequence of isolate 7 shared on BLAST analysis 98.0% homology with that of a strain isolated from the lower respiratory tract of a cystic fibrosis patient (35) and 96.3% with *Chryseobacterium scophtalmum* strain LMG 13028^T (Table). On genetic analysis, isolate 7 shared 92.5%–97.1% 16S rDNA sequence homology with *Chryseobacterium* spp. and 81.8%–90.3% with other *Flavobacteriaceae*, respectively. Phylogenetic analysis of the 16S rDNA gene (Figure 3) suggested that isolate 7 represented a new species of *Chryseobacterium* (Figure 3). Thus, bootstrap values of 100%, 100%, and 97% in neighbor-joining, minimum evolution, and parsimony analyses, respectively, supported the fork's separating isolate 6 from its closest neighbor, an unpublished species of *Chryseobacterium*. The morphologic features of isolate 7 within *A. polyphaga*, as seen by electron microscopy, is shown in Figure 2. Isolate 7 has been deposited in the Collection de l'Institut Pasteur, Paris, France, as *Candidatus Chryseobacterium massiliae* strain CIP 107752^T.

Discussion

Recovery of *Methylobacteriaceae*, *Rhizobiaceae, Alcaligenaceae*, and *Flavobacteriacae* by amoebal coculture shows that these clades should be added to the growing number of ARB. Our study also shows that humans are exposed to unknown ARB and that the amoebal coculture is an effective tool for the recovery of new species from contaminated samples.

Until this study, the ARB included *Legionella* spp. (4,36), *Chlamydiales* (3,5), *Bradyrhizobiaceae* (37), *Rickettsiales* (38), *Listeria monocytogenes* (39), *M. avium* (40), *Procabacter acanthamoeba*, a betaproteobacteria (41), and members of the Bacteroides-Cytophaga-Flexibacter group (*Flavobacterium* spp. and *Amoebophilus asiaticus*) (42). In this study, we recovered additional alphaproteobacteria including a strain of *Methylobacterium extorquans*, a *Bosea* sp., a new *Rhizobium* sp., provisionally named *Candidatus Rhizobium massiliae*, and a new *Roseomonas* sp., named *C. Roseomonas massiliae*. We also recovered a strain of *Achromobacter xylosoxidans* (betaproteobacteria) and two new *Flavobacteriacae* related to the *Chryseobacterium* spp., provisionally named *C. Chryseobacterium massiliae* and *C. Amoebinatus massiliae*. Whether these species use the free-living amoebae as a reservoir and whether they play a role as human pathogens remain to be defined.

ARB in nasal passages of patients and homeless persons are important because they demonstrate the exposure of humans to these bacteria. From the nasal mucosa, ARB may spread to the lower respiratory tract, where they might cause bronchitis and pneumonia, being somewhat adapted to intracellular life. The fact that ARB were more likely to be recovered from patients hospitalized in the infectious disease department than from the other patients sampled is of interest. Further studies will be needed to confirm this statistically significant association and to determine whether patients were colonized with ARB during their hospitalization, whether colonization with ARB may be a marker of exposition to another pathogen involved in the infectious process that lead to hospitalization, or whether the isolated ARB may itself be the etiologic agent of the infectious process that led to hospitalization.

Our study also shows that amoebal coculture is a cell culture system that may be used to recover new bacterial species from heavily contaminated clinical or environmental samples. Indeed, by grazing on bacteria, the free-living amoebae will clean the samples from most rapid growing species that generally overwhelm the agar plates (for example *Enterobacteriaceae, Staphylococci*, *Streptococci*, and *Neisseriaceae* in a nasal sample). Thus, using that technique, Rowbotham was able to grow *L. pneumophila* from human feces (43). Moreover, the amoebal coculture is a cell culture system that may be performed in the absence of antimicrobials and is thus especially suitable for recovery of new bacterial species of unknown antimicrobial susceptibility. Relative to a broad amplification strategy for microbial rDNA found in nasal washings, amoebal coculture has several advantages. First, it makes a strain available; its phenotype, genotype, antibiotic susceptibility, and virulence may then be further characterized. Second, a broad amplification strategy is unlikely to be successful in obtaining a clear sequence from such heavily contaminated sample. As amoebal resistance may

be associated with virulence (11–16), the amoebal coculture might have the additional advantage of selectively growing potential human pathogens. The main limitations of this technique are the decreased viability of the amoebal cells and their encystment at high incubation temperature, which do not allow the recovery of bacteria requiring a temperature ≥37°C. Another limitation is that, contrary to amoebal enrichment, amoebal coculture did not recover the amoebal host, if any, present in the sample. In addition, this method, like other cell cultures, is associated with a substantial laboratory workload.

We did not isolate *Parachlamydia* spp., which may reflect the scarcity of sources or the low or nonexistent exposure to this potential human pathogen in the area where the survey was performed. This finding may also explain the low prevalence of *Parachlamydia*-associated community-acquired pneumonia that we observed in a recent study (44).

In conclusion, amoebal coculture allows new bacterial species to be isolated from heavily contaminated human samples and might be a valuable approach in identifying potential, as-yet unrecognized human pathogens from nonsterile clinical samples, such as upper and lower respiratory tract specimen.

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Dr. Greub is a specialist in medical microbiology and infectious disease. He currently works at the Institute of Microbiology of the University of Lausanne, Switzerland. His research focuses on *Parachlamydia acanthamoebae* and other emerging intracellular bacteria.

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Neutralizing Antibodies and Sin Nombre Virus RNA after Recovery from Hantavirus Cardiopulmonary Syndrome

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Patients who later have a mild course of hantavirus cardiopulmonary syndrome (HCPS) are more likely to exhibit a high titer of neutralizing antibodies against Sin Nombre virus (SNV), the etiologic agent of HCPS, at the time of hospital admission. Because administering plasma from patients who have recovered from HCPS to those in the early stages of disease may be an advantageous form of passive immunotherapy, we examined the neutralizing antibody titers of 21 patients who had recovered from SNV infection. Even 1,000 days after admission to the hospital, 6 of 10 patients had titers of 800 or higher, with one sample retaining a titer of 3,200 after more than 1,400 days. None of the convalescent-phase serum samples contained detectable viral RNA. These results confirm that patients retain high titers of neutralizing antibodies long after recovery from SNV infection.

Hantaviruses are negative-stranded RNA viruses of the family *Bunyaviridae*. At least 11 members of the rodent-borne genus *Hantavirus* have been associated with hantavirus cardiopulmonary syndrome (HCPS) or hemorrhagic fever with renal syndrome (HFRS) in humans (1). Sin Nombre virus (SNV), the prototype etiologic agent of HCPS, is carried by the deer mouse, *Peromyscus maniculatus* (2). While four other etiologic viruses cause HCPS in North and Central America and at least two cause HPCS in South America, SNV accounts for most of the >300 known North American cases.

SNV is transmitted primarily by inhalation of contaminated aerosols of rodent urine, feces, or saliva. The first symptoms appear 9–33 days later (3). After a prodromal phase of 1 to 6 days, consisting of fever, myalgia, headache, malaise, gastrointestinal disturbances, and thrombocytopenia, hypotension or shock and acute pulmonary edema develop in most patients (4). In practice, HCPS is provisionally diagnosed in most patients, and they are admitted to a hospital on the first day that pulmonary edema occurs. In patients with fatal cases, death occurs within 3 days after the onset of respiratory symptoms. Because such a narrow window exists between presentation and lethal outcome, improving the outcome will likely require rapid and decisive intervention, perhaps before the ultimate severity of the disease is known for a particular patient. Since most deaths are caused by myocardial dysfunction and hypoperfusion rather than hypoxia, some investigators have recently begun to use the term hantavirus cardiopulmonary syndrome (HCPS) rather than the previous term hantavirus pulmonary syndrome.

Antibodies of at least the immunoglobulin (Ig) M class are present from the earliest clinical stages of HCPS, and IgG antibodies against either the nucleocapsid (N) or G1 glycoprotein antigen are present in most patients even in the prodrome phase (5). Recently, we examined the kinetics of the development of antibodies capable of in vitro neutralization of SNV in patients with HCPS and found that many patients had exceptionally high titers (≥800) of such antibodies from the first day of clinical illness (6). In addition, we found that patients who had a milder course of disease had markedly higher titers of neutralizing antibodies on admission than did those patients who later exhibited a more severe infection. Because other acute viral infections have been successfully treated with the plasma of patients who had recovered from these diseases, we are contemplating the use of such treatment for patients with HCPS. Toward this end, we examined the kinetics of the decay of neutralizing antibodies in patients who had recovered from SNV infection 3 months to 5 years before.

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Materials and Methods

Study Participants

Patients were considered to have acute SNV infection on the basis of the following serologic criteria: the presence of IgM and IgG antibodies directed against the SNV N antigen and the presence of IgG antibodies against the viral G1 antigen. The latter marker is specific for infection with SNV (7). A total of 21 samples were collected from 21 patients who were called back for reevaluation as part of a study of the sequelae of HCPS caused by SNV (D. Goade, unpub. data). Informed consent was obtained from patients or their parents or guardians, and human experimentation guidelines of the U.S. Department of Health and Human Services and the University of New Mexico Human Research Review Committee were followed in the conduct of this research.

Focus Reduction Neutralization Test (FRNT)

The serum samples from HCPS patients were examined by FRNT in at least duplicate analyses in 48-well tissue culture plates (6). (We did not subject serum samples to heat inactivation because previous studies had shown that decomplementation did not significantly change the measured FRNT titers of a group of human or rodent serum specimens with titers between 800 and 1,280 [C. Ye, unpub. data].) Samples were serially diluted (1:50, 1:100, 1:200, 1:400, 1:800, 1:3,200, 1:12,800) and mixed with equal volumes of approximately 45 focus-forming units (ffu) of SNV strain SN77734 (8) for 1 h at 37°C before incubation on Vero E6 cells. The dilution buffer consisted of complete minimal essential medium (MEM; Gibco/BRL, Grand Island, NY) containing 2.5% fetal bovine serum (HyClone Laboratories, Logan, UT). After adsorption for 1 h at 37°, the cells were washed in phosphate-buffered saline (PBS) and overlaid with medium containing 1.2% methylcellulose for 7 days. The methylcellulose layer was removed, and the cells were fixed with 100% methanol with 0.5% hydrogen peroxide. Viral antigen was visualized by the addition of hyperimmune rabbit anti-SNV N protein (1:5,000), followed by peroxidase conjugated goat anti-rabbit IgG (Jackson Immuno Research Laboratory, West Grove, PA) and DAB/metal concentrate as substrate (Pierce Chemicals, Rockford, IL). The neutralization activity of a patient's serum specimen was expressed as the maximum serum dilution that would reduce the number of viral foci by 80% or more.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

RT-PCR was conducted as described previously (8). The nested protocol in question has a sensitivity of <10 copies of viral RNA per reaction when RNA is isolated from cell-free materials such as serum (J. Botten, B. Hjelle, unpub. data). The primers, which have been described previously (8), were located near the 5′ terminus of the gene encoding the SNV N antigen. Many sequences spanning this region have been examined from SNV isolates from throughout the United States and Canada, and little variation has been detected (B. Hjelle, unpub. data). In each case, RNA was prepared from 100 μL of serum and 20% of the preparation was subjected to nested RT-PCR analysis.

Results

The Table shows the geographic origin, age range, sex, and clinical score of each of the 21 patients. Patients traveled to Albuquerque, New Mexico, as funds permitted from the University of New Mexico's Research Allocation Committee. The patients were subjected to a battery of clinical tests, including studies examining pulmonary, renal, and hepatic function. The results of the clinical studies will be reported separately.

Serum samples were collected for virologic studies and examined for residual viral RNA by RT-PCR and for neutralizing antibodies. Although positive control RNA preparations produced amplification products as expected, no viral RNA was detected in any of the patients' serum samples (data not shown). However, the serum samples examined continued to exhibit neutralizing antibodies at titers ranging from 100 to 3,200 (Figure). No statistically significant relationship existed between neutralizing antibody titers after the patient's recovery from SNV infection and the severity of the previous illness, nor did a relationship exist between antibody titers and the use of extracorporeal membrane oxygenation (ECMO) in our patient group.

Discussion

Although case-fatality ratios for HCPS have declined since the initial description of the disease, probably reflecting improved recognition of mild illness, therapeutic options remain limited. The efficacy, if any, of antiviral drugs such as ribavirin remains to be demonstrated. Intensive care management of the manifestations of the disease constitutes the primary method of clinical intervention. At some centers, ECMO has been utilized in those patients who exhibit hemodynamic or pulmonary instability that is predictive of fatal outcome (10). (Six of our patients underwent this procedure.)

Although treating patients with viral hemorrhagic fevers with the plasma of patients who have recovered from the disease is often proposed or discussed, rigorous demonstration of its efficacy in humans or animals is often lacking. Argentine hemorrhagic fever (AHF) due to Junín virus and other arenaviral diseases are among the exceptions (11–14). The efficacy of convalescent-phase plasma

Table. Demographic and clinical characteristics of 21 patients with history of Sin Nombre virus infection

Patient	Age at time of illness	Sex	Date admitted	Day serum collected	Location	Severity[b]
1	10	M	30 Mar 1999	589	NM	I
2	44	F	4 Dec 1995	1789	NM	IIE
3	28	F	5 Apr 1999	592	NM	0
4	28	F	3 Apr 1999	566	WA	II
5	42	M	15 Jun 1999	94	NM	I
6	29	F	1 May 1998	1063	NM	I
7	18	F	11 Aug 1998	512	CO	IIE
8	44	F	29 Mar 1994	1627	NM	II
9	43	M	1 Sep 2000	231	AZ	IIE
10	41	M	20 Mar 1997	1436	KS	II
11	39	F	6 Mar 2001	85	NM	II
12	50	M	20 Aug 1998	334	NM	IIE
13	34	F	15 Jul 1999	92	CO	I
14	44	F	3 Nov 1997	1281	KS	II
15	19	F	18 Oct 2000	132	MT	II
16	27	M	23 Jul 1997	1356	NM	I
17	27	F	27 Jan 1998	1196	KS	I
18	23	F	30 Oct 1999	161	NM	IIE
19	16	M	19 Sep 1997	1419	TX	II NO (9)
20	21	F	27 May 1994	2268	NM	II
21	44	M	20 Dec 1997	1056	WI	II

[a]F, female; M, male; NM, New Mexico, WA, Washington, CO, Colorado; AZ, Arizona; KS, Kansas; MT, Montana; TX, Texas; WI, Wisconsin.
[b]Severity scale: Class 0, prodromal symptoms with seroconversion but with no cardiopulmonary manifestations; Class I, prodrome symptoms with hypoxia but not requiring endotracheal intubation; Class II, survived but required endotracheal intubation; Class IIE, required extracorporeal membrane oxygenation; II NO, treated with inhalational nitric oxide. Because they required endotracheal intubation, patients in Class II are regarded as having been more severely ill than those in Classes 0 and I.

in treating AHF was demonstrated before the nature of the protective substance contained therein was positively identified. With the advent of a test for neutralizing activity, Argentine investigators retrospectively demonstrated a correlation between the quantity of the neutralizing activity of plasma and the degree of protection afforded by its infusion (12).

The neutralizing activity of a dose of plasma is defined by multiplying the reciprocal of the highest dilution that would reduce the number of viral foci by 80% by the volume of the inoculum, in milliliters. Overall, the infusion of convalescent-phase plasma containing antibodies to Junín virus reduced deaths by approximately 90%. In a retrospective analysis, investigators showed that those patients who received >2,000 U/kg of neutralizing antibodies appeared to respond better than did those patients who received 1,000–2,000 U (12).

Because HCPS is rare, the number of treatments that can be independently examined through clinical trials without delaying the identification of an effective intervention is inherently limited. To determine whether a clinical rationale exists for attempting passive immunotherapy for HCPS with neutralizing antibodies, we first studied the relationship between the titer of such antibodies on a patients' admission to the hospital and the clinical outcome. That study showed a profound and inverse relationship between antibody titer and disease severity (6).

It then became important to determine whether the small number of patients who have recovered from SNV infection might constitute a pool of plasma donors of sufficient size to provide a stable source of plasma to treat future patients. The study reported herein was initiated to determine whether neutralizing antibodies persist for substantial periods after recovery from the disease. Results indicate that the answer is affirmative but that not all recovered patients are equally rich sources of neutralizing antibodies.

Modern techniques for collecting plasma allow for convenient and low-risk preparation of 500–600 mL or more of plasma from healthy donors; this can be repeated on multiple occasions over several weeks. For that and other reasons, each of those patients whose titers remained at $\geq$800 represents a rather rich source of antibodies. If one extrapolates from the experience of the Argentine investigators in their treatment of AHF, a single 600-mL plasma donation by a person with a neutralizing antibody titer of 800 can be used to treat two 80-kg (~175-lb) patients. A patient such as the person whose titer was 3,200 could be identified as a particularly valuable repeat donor, one whose every donation could be divided and used to treat eight new patients with HCPS.

Thus far, little or no overlap occurs among the behavioral characteristics associated with HCPS and those associated with infection by bloodborne pathogens such as HIV or hepatitis C virus. Potential donors would be subjected to the same battery of questions and tests for bloodborne pathogens as would any other directed blood donors, with the exception that donors would be allowed to donate despite the history of hospitalization for HCPS and receipt of blood products during ECMO therapy. Although we

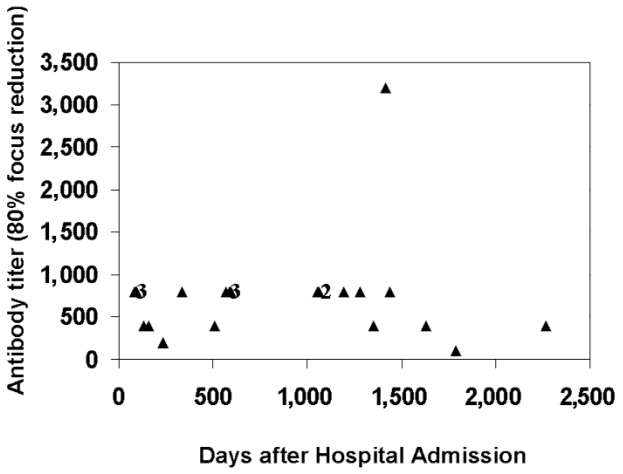

Figure. Titers of neutralizing antibodies against Sin Nombre virus (SNV) strain SN77734 in serum samples from patients surviving hantavirus cardiopulmonary syndrome due to SNV. The reciprocal of the endpoint neutralization titer is plotted for each sample. Numbers near certain clusters of points reflect the number of individual data points represented in a particular cluster.

therefore expect that most potential donations of convalescent-phase plasma would be free of bloodborne pathogens, ensuring that SNV has been completely cleared from the blood is more difficult. Thus far, no evidence for a persistent state of SNV infection in humans has been shown. In fact, studies published to date show that in all cases that could be evaluated, SNV is undergoing rapid clearance at the time of clinical presentation (15).

Although our RT-PCR data are reassuringly negative, we cannot know whether a larger dose of convalescent-phase plasma would contain infectious SNV. In fact, SNV has never been cultivated from any human source, despite attempts by multiple laboratories.

We believe that passive immunotherapy may be efficaceous if administered very early in the course of illness, before the severity of a particular patient's disease course is known. To reduce the risk for accidental inoculation of potential recipients of anti-SNV convalescent-phase plasma, at least two strategies are possible. First, one could impose a delay of 6 to 36 hours by requiring that potential recipients have a positive specific test for SNV antibodies before the plasma is administered. Alternatively, one could impose either purification or viral inactivation procedures for the antibodies or await the development of human or humanized monoclonal antibodies before instituting this therapy. We presume in our risk analysis that no new risk is associated with the inadvertent inoculation of a second strain of SNV in a patient who is undergoing active infection with a different genotype of the same virus, since there is no a priori evidence to suggest otherwise.

No current evidence suggests that infusing anti-SNV antibodies would enhance the pathogenesis of SNV. The data of Bharadwaj et al. (6), in fact, support the presumption that treatments that lead to higher titers of neutralizing and even nonneutralizing anti-SNV antibodies are more likely to lead to less severe disease. We believe that convalescent-phase plasma therapy may represent a practical intervention that is feasible, given current resources and funding levels for potential antihantavirus therapies.

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First Reported Prairie Dog–to-Human Tularemia Transmission, Texas, 2002

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A tularemia outbreak, caused by *Francisella tularensis* type B, occurred among wild-caught, commercially traded prairie dogs. *F. tularensis* microagglutination titers in one exposed person indicated recent infection. These findings represent the first evidence for prairie-dog-to-human tularemia transmission and demonstrate potential human health risks of the exotic pet trade.

Tularemia is a zoonosis affecting more than 150 wildlife species, including prairie dogs, squirrels, cats, and humans (1–3). Tularemia is caused by the bacterium *Francisella tularensis*, which exists in two main types. Type A is found almost exclusively in North America and is highly virulent in humans. Type B exists throughout North America, Asia, and Europe and is less virulent in humans (4). Tularemia vaccines have been used to protect military and laboratory personnel at high risk for exposure but are not available for the general population (5).

Humans can acquire tularemia through contact with infected animals (2,3,6). Although not previously documented, transmission to humans from prairie dogs is a concern because thousands of wild prairie dogs are captured annually in the United States and sold as exotic pets worldwide (7).

In mid-July 2002, a die-off began among wild-caught, black-tailed prairie dogs (*Cynomys ludovicianus*) (Figure 1) at a commercial exotic pet distributorship in Texas (facility A). On July 29, one of the dead prairie dogs tested positive for *F. tularensis* (8). Hundreds of potentially infected prairie dogs had already been distributed to other states and exported internationally. Epidemiologic and microbiologic investigations were initiated on August 1. We report on the epidemiologic findings; the microbiologic investigation is reported separately (9).

The Study

Animal Investigation

Facility A's purchasing and shipping records were reviewed and the staff interviewed. All involved states and countries were notified of the outbreak, asked to identify the status of prairie dogs from the suspected shipments, and submit tissue samples for testing.

All prairie dogs at facility A, prairie dogs distributed within Texas from facility A since June 2002, and other dead and free-roaming exotic species at facility A were retrieved; live animals were euthanized; and all were tested for *F. tularensis* by direct fluorescence assay (DFA) and culture on cysteine heart agar with 9% chocolatized blood media (9). All recovered isolates were subtyped by using a polymerase chain reaction (PCR) assay (9).

Trappers who supplied prairie dogs to facility A in May and June 2002 were interviewed, and prairie dogs from their respective facilities in Texas and South Dakota were euthanized and tested for tularemia. South Dakota trapping sites suspected to be a potential source of the outbreak were also investigated.

Investigation of facility A on August 2 indicated a variety of exotic species crowded within a 2,500 square foot building. We found 163 remaining prairie dogs in four groups: sick and dying prairie dogs (bin 1), healthy-appearing prairie dogs (bin 2 and cages), prairie dog carcasses (frozen), and escaped prairie dogs roaming free

Figure 1. Black-tailed prairie dogs (*Cynomys ludovicianus*).

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around the facility. The bins were metal, uncovered, 2.5 feet tall and 5 feet in diameter, with 50–100 prairie dogs per bin. In addition, several other exotic animals were found roaming free or dead.

According to shipping records, approximately 3,600 prairie dogs passed through facility A during January through July 2002. In July, an estimated 250 prairie dog deaths occurred compared with approximately 25 deaths over the previous 6 months (Figure 2). On August 1, shipments to and from facility A were halted.

Necropsies on all 163 prairie dogs remaining in facility A indicated clinical signs of oropharyngeal tularemia in all the dead and most of the euthanized sick animals, suggesting transmission through ingestion. Many of the dead animals had been cannibalized. *F. tularensis* was isolated from 61 animals (Table 1). Of these, 60 isolates came from prairie dogs remaining in facility A, including one prairie dog roaming free in the facility, and one isolate came from a privately owned prairie dog purchased from a Texas pet shop supplied by facility A. All of the isolates were identified as type B.

During June through July 2002, more than 1,000 prairie dogs were distributed from facility A to locations in 10 U.S. states and 7 other countries (Table 2). By early August, 100 prairie dogs, those shipped to the Czech Republic, remained unsold: of these, approximately 30 were dead on arrival, 30 were ill, and evidence of cannibalism had been noted within the shipment. All living animals were euthanized.

Of the prairie dogs distributed from facility A to other U.S. states, specimens were received from two prairie dogs sent to Michigan; serum samples from both tested negative for tularemia (Table 1). The Netherlands and Belgium retrieved 4 and 10 prairie dogs, respectively, for serologic testing and culture of tissue samples; all were reported to be negative. The Czech Republic tested six prairie dogs for tularemia: one was positive by isolation of *F. tularensis* in culture, and five were presumptively positive by polymerase chain reaction (PCR). The Czech *F. tularensis* isolate was identified as type B, indistinguishable from the Texas isolates by restriction fragment length polymorphism analysis (9).

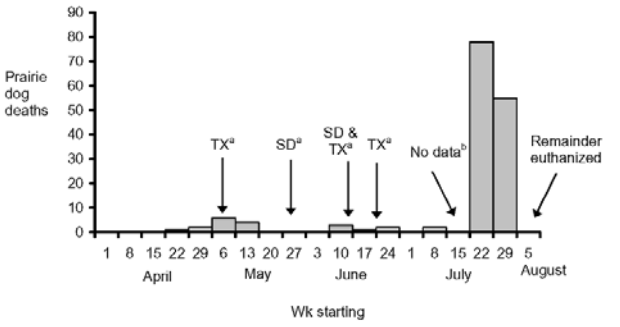

Figure 2. Weekly prairie-dog deaths at facility A, Texas, April–August, 2002. [a]Arrows represent prairie dog shipments arriving at facility A from Texas (TX) and South Dakota (SD).[b]No data are available for the week of July 15, when the outbreak was first noticed by facility A staff.

All healthy-appearing prairie dogs in bin 2 and cages, as well as other exotic animals roaming free or found dead in facility A tested negative for tularemia, demonstrating that outbreak propagation required direct contact with infected prairie dogs. Prairie dogs collected from Texas trappers, South Dakota trappers, and trapping sites all tested negative.

Human Investigation

A human case was defined as a fourfold change in serial *F. tularensis* antibody titers from serum samples obtained at least 14 days apart, with at least one titer ≥1:128, in an exposed person. Paired serum samples were tested with an *F. tularensis* microagglutination assay. Anyone who transported, handled, bought, or cleaned the cages of prairie dogs from facility A since June 2002 was considered exposed. Exposed persons in Texas and other U.S. states were given a standardized questionnaire to assess infection risk factors and symptoms during the 2 weeks after their exposure. To enhance case finding, periodic follow-up was maintained with health authorities in involved U.S. states and foreign countries.

Table 1. Diagnostic results for all animals tested in association with tularemia outbreak in prairie dogs, Texas, 2002

Location	Species	No. animals tested	Confirmed positive[a]
Facility A	Prairie dogs	163	61
Retrieved from other Texas facilities	Prairie dogs	7	1
Czech Republic	Prairie dogs	6	1
Trapper facility, TX	Prairie dogs	8	0
Trapper facility, SD	Prairie dogs	2	0
Michigan	Prairie dogs	2	0
Facility A	Chinchilla, sugarglider, hedgehog, red squirrel, eastern chipmunk	16	0
Field investigation, Mellette County, SD	Prairie dogs, deer mice, white-footed mice, grasshopper mice, ground squirrel, jack rabbit, meadow vole	90	0

[a]Prairie dogs were confirmed positive on recovery of an isolate with characteristic growth on cysteine heart agar with 9% chocolatized blood and positive testing of the isolate by direct fluorescent antibody or polymerase chain reaction.

Table 2. Numbers of prairie dogs distributed from facility A to U.S. states and countries in Europe and Asia, June–July, 2002

Locations	No. prairie dogs
United States	
Texas	115
Illinois	26
Ohio	20
Washington	18
Arkansas	12
Nevada	12
West Virginia	12
Michigan	2
Florida	1
Mississippi	1
Europe	
the Netherlands	400
Belgium	250
Czech Republic	100
France	2
Portugal	1
Asia	
Japan	328
Thailand	2

Twenty-two exposed persons were identified in Texas: 5 worked at facility A, 13 worked at other Texas facilities supplied by facility A, 3 worked at a veterinary care center and necropsied a prairie dog originating from facility A, and 1 privately owned an infected prairie dog originating from facility A. In interviews with 20 of 22 exposed persons, 6 (32%) reported recent prairie-dog bites, 7 (37%) ate or drank without handwashing after contact with prairie dogs, and 13 (67%) handled prairie dogs or cleaned cages barehanded. Although gloves and soap were available to employees, none of the involved Texas facilities had formal written policies enforcing proper handwashing, wearing gloves, or prohibiting eating or drinking in animal care areas.

During their exposure interval, 14 of 20 exposed persons interviewed reported having ≥2 nonspecific symptoms that can be consistent with tularemia: headache, sore throat, myalgias, stiff neck, fever, chills, cough, and swollen glands. Initial serologic testing on blood samples obtained 1 week to 2 months after initial exposure from 19 of 22 persons in Texas identified a positive *F. tularensis* titer of 1:128 in a 24-year-old man, who was an animal handler at facility A. All other persons tested negative, and no new positive titers were identified from follow-up samples obtained 1–2 months later from 9 of 19 persons. Except for the animal handler, other symptomatic persons had spontaneous resolution of symptoms or other diagnoses for their symptoms. The animal handler's 1-month follow-up titer persisted at 1:128; however, a fourfold decline in titer, from 1:128 to 1:32, was documented for samples obtained 4 and 6 months after the initial titer, indicating recent exposure to *F. tularensis*. The animal handler had begun working at facility A in June 2002 and had handled dead and dying prairie dogs barehanded. He denied prior potential tularemia exposures, such as hunting, having tick bites, or owning a pet. Additionally, he denied having received a tularemia vaccine, which could have explained the elevated titer. During our investigation, the animal handler reported having an afebrile upper respiratory infection-like illness atypical of tularemia, with sore throat, cough productive of green sputum, and mild chest discomfort but no interruption of work or leisure activities. His symptoms began 12 days after the last prairie dog shipment arrived at facility A and 1 week before the die-off, and they resolved after oral fluoroquinolone therapy.

Health authorities in other states and countries reported no illness in exposed persons. Six months after the outbreak occurred, follow-up calls to health authorities in the involved U.S. states indicated no new human cases. No serologic testing was performed on exposed persons outside of Texas.

Conclusions

Our investigation demonstrated the first evidence that prairie dogs can transmit tularemia to humans. The animal handler's atypical symptoms and unclear route of infection might be because he was exposed to the less virulent subspecies type B. Studies have documented higher rates of *F. tularensis* seropositivity among animal trappers from tularemia-endemic areas, and many of the trappers were asymptomatic (10).

This outbreak highlights health risks to humans who handle wild-caught animals and underscores the speed with which exotic species and virulent pathogens can be transported worldwide (11). A number of public health risks associated with the exotic pet trade were observed at facility A. Prairie dogs were crowded in large bins, allowing unnaturally close contact and propagation of the outbreak through cannibalism. A variety of wild-caught and captive-bred exotic animals were also held in close quarters, providing opportunity for diseases to jump species. This risk for disease transmission between species was heightened because several exotic animals were able to roam free and comingle.

Until recently in the United States, no federal regulations existed to protect humans from the domestic distribution and sale of infected, wild-caught animals; a ban against transport and sale of prairie dogs and certain other rodent species was implemented on June 11, 2003, in response to a monkeypox outbreak in the Midwest (12). Many states forbid capture and sale of native wildlife species, including prairie dogs; however, states that do permit trapping and sale do not have regulations to address the human risk of acquiring zoonoses.

This incident and others, such as transmission to humans of plague from prairie dogs, monkeypox from prairie dogs, and salmonellosis from African pygmy hedgehogs, highlight the importance of developing strategies to reduce human risk from the domestic and international sale of infected, wild-caught animals (13–16). Strategies might include educating the public, standardizing exotic animal husbandry practices, restricting trade to animals bred in captivity, or banning sale of wild-caught animals. As a result of this investigation, Japan banned prairie dog importation as of March 2003. We recommend that the United States and other countries review and strengthen their regulations governing the transport and sale of prairie dogs and other exotic pets.

Acknowledgments

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Predicting Quarantine Failure Rates

Troy Day*

Preemptive quarantine through contact-tracing effectively controls emerging infectious diseases. Occasionally this quarantine fails, however, and infected persons are released. The probability of quarantine failure is typically estimated from disease-specific data. Here a simple, exact estimate of the failure rate is derived that does not depend on disease-specific parameters. This estimate is universally applicable to all infectious diseases.

Preemptive quarantine (isolating asymptomatic persons who have had contact with an infected person) is an effective technique for slowing the spread of emerging infectious diseases, but it also results in many uninfected persons being isolated (for examples, see [1,2]). Health officials must determine an acceptable quarantine duration that balances the social and financial costs of holding potentially uninfected persons for long durations with the risk of releasing an infected person into the general public before he or she displays symptoms, if a shorter duration is used (the quarantine failure rate, ϕ). One primary consideration in setting the quarantine duration is the range of observed incubation times. Often the quarantine duration is set to be (approximately) equal to the longest observed incubation period in a sample of n infections (3). The quarantine failure rate is then monitored through the collection of data on incubation periods throughout the outbreak (3).

This approach requires considerable effort, and it must be carried out for each new disease. This assessment of quarantine failure rates is also necessarily retrospective, with the data required for analysis becoming available only after the fact. Here a much simpler approach is derived that requires no data specific to the disease in question. It applies for all possible infectious diseases, and therefore can be employed proactively rather than retrospectively.

If the quarantine duration is chosen to be the longest incubation period in a sample of n infections, then the probability, χ, that the quarantine failure rate is no larger than π, is

$\chi = 1 - (1 - \pi)^n$ (equation 1)

for all possible infectious diseases (Appendix). For example, the probability that the quarantine failure rate is no larger than 1% is simply $\chi = 1 - (1 - 0.01)^n$. This is valid irrespective of any of the biologic details of the disease of interest. In particular, the form of the underlying probability distribution of incubation times for the disease at hand has no influence on this result.

Often it is of more interest to estimate the quarantine failure rate at a prescribed level of certainty. By rearranging equation 1, we have: with χ% certainty, the quarantine failure rate, φ, is no larger than π, where

$\pi = 1 - (1 - \chi)^{1/n}$. (equation 2)

For example, the 95% confidence boundary for the failure rate is simply

$\pi = 1 - (1 - 0.95)^{1/n}$. Moreover, a point estimate for the failure rate is obtained by calculating the expectation of ϕ:

$$\bar{\phi} = \frac{1}{n+1}$$.(equation 3)

Indeed, more generally the probability density of quarantine failure rate, ϕ, is simply $p(\phi) = n(1 - \phi)^{n-1}$ for any infectious disease (Appendix).

The above results also allow one to evaluate the protocol of using the largest incubation period of n infected hosts as the quarantine duration. For example, if $n = 35$ (a reasonable value for a newly emerging disease) then the point estimate for ϕ is (equation 3) 1/36 (2.8%), and we have 95% confidence that ϕ is no larger than 8.2% (equation 2). Thus, a failure rate of 8 in 100 infected persons inadvertently being released from quarantine is within the 95% confidence region. This failure rate is likely unacceptable for highly transmissible diseases.

Alternatively, the above results can be used to determine the sample size, n, on which the quarantine duration must be based to ensure that the quarantine failure rate is less than π with χ% certainty. Rearranging equation 1 yields

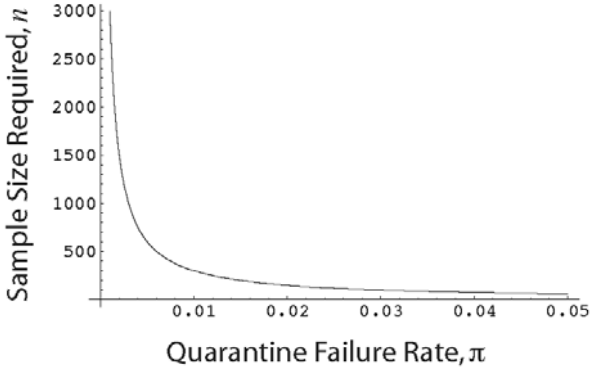

Figure. Sample size of infections, n, that the quarantine duration must be based on to ensure that the quarantine failure rate is no larger than π (with 95% certainty). Results assume that the quarantine duration is set equal to the largest incubation period observed in the sample of n infections. Curve is plotted using equation 4 with $\chi = 0.95$.

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$$n = \frac{\ln(1-\chi)}{\ln(1-\pi)} \quad \text{(equation 4)}$$

This is plotted in the Figure, indicating that enormous sample sizes are required to ensure that the quarantine failure rate is <1%. Together, the above results therefore call for two amendments to preemptive quarantine protocols. First, update the quarantine duration as further infections are observed during an outbreak. This amendment keeps *n* as large as possible. Secondly, set the quarantine duration to be longer than the maximum observed incubation period during the initial stages of the epidemic, when the sample size, *n*, is necessarily small.

Dr. Day is a mathematical biologist conducting theoretical research in evolutionary biology. One of his primary interests is in developing theory to better understand and predict the evolutionary and epidemiologic dynamics of infectious diseases.

Appendix: Explanation of Formula for Predicting Quarantine Failure Rates

Let L be a random variable denoting the incubation period, and $f(l)$ and $F(l)$ be its probability density function (p.d.f.) and cumulative distribution function. Let M be a random variable denoting the maximum incubation period in a sample of n infections. The p.d.f. of M is then given by $nf(m)F(m)^{n-1}$. In other words, the probability that, after n draws the maximum incubation period is m, is given by the product of the probability that one draw yields an incubation period of exactly m (i.e., $nf(m)dm$) with the probability that the remaining $n-1$ draws all yield incubation periods no larger than m (i.e., $F(m)^{n-1}$). Now introduce a new random variable, $X = F(m)$ (lying in [0,1]), representing the probability that an infected host will have an incubation period no larger than m (where F is the same cumulative distribution function introduced above). The p.d.f. of X is

$$\frac{d}{dx}\int_0^{G(x)} nf(m)F(m)^{n-1}dm = nx^{n-1},$$

where $G(x)$ is defined to be the inverse of $F(x)$. The quarantine failure rate is $1-X$, and therefore its p.d.f., $p(\phi)$, is

$p(\phi) = n(1-\phi)^{n-1}$. We then also have

$$\chi \equiv \int_0^{\pi} n(1-\phi)^{n-1}d\phi = 1-(1-\pi)^n \quad \text{and}$$

$$\bar{\phi} \equiv \int_0^1 \phi n(1-\phi)^{n-1}d\phi = 1/(n+1).$$

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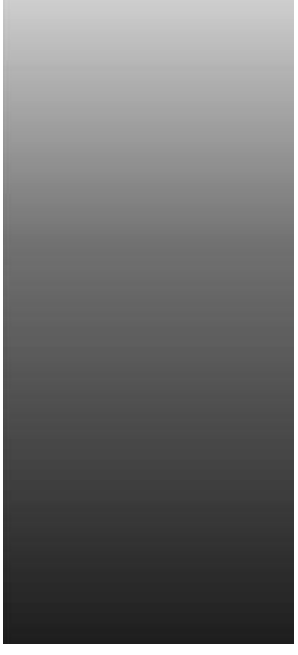

Patient Data, Early SARS Epidemic, Taiwan

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Of the first 10 patients in the epidemic of severe acute respiratory syndrome (SARS) in Taiwan, 4 were closely associated with a SARS patient in an airplane. Loose stools or diarrhea, hemophagocytosis syndrome, and high serum levels of interleukin (IL)-6, IL-8, and tumor necrosis factor-α associated with lung lesions were found in all 10 patients.

In November 2002, severe acute respiratory syndrome (SARS), a highly contagious febrile respiratory illness with a high propensity to spread to a patient's household members and healthcare workers, emerged and eventually caused worldwide outbreaks (1–9). By late February 2003, SARS was considered an emerging disease (10). Most SARS cases have occurred in China, the Hong Kong Special Administrative Region, Canada, and Taiwan (1–9). As of July 15, 2003, there were 671 probable cases of SARS in Taiwan; 84 were fatal.

The Study

From March 8 to April 16, 2003, the first 10 patients whose illness met the World Health Organization (WHO) case definition of probable SARS (10) were admitted to National Taiwan University Hospital. Three patients (patients 6, 7, and 8) were transferred to other hospitals after initial evaluation and treatment at the emergency department.

All 10 patients were Taiwanese; 7 had recently returned to Taiwan from China (2 from Guangdong Province, 5 from Beijing), 2 were family members, and 1 was a healthcare worker. Among the 10 patients, two clusters involving 9 patients were identified. Transmission among the four patients of cluster A occurred after household contact with the index case-patient (patient 1) or contact with patient 2 in a healthcare setting (Figure 1A). Transmission between the five patients of cluster B was associated with close contact with a SARS patient (patient X) on an airplane (Figure 1B). Patient 9 did not fly with the four patients but had contact with them in Beijing, particularly with patient 5.

Clinical features and laboratory characteristics of the 10 SARS patients are summarized in the Table. The patients were 26–53 years old (mean 42.7 years); eight were men. All of these patients were previously healthy. The incubation period was 2–12 days. Loose stools or diarrhea developed within 2 to 10 days in eight patients after the onset of fever. Acute respiratory distress syndrome necessitating mechanical ventilation developed in four patients (patients 1, 2, 3, and 9). All but one patient (patient 6) had high C-reactive protein (CRP) values (range 21.9 mg/L–>120 mg/L) at admission. Among the seven patients whose serum ferritin and creatinine phosphokinase levels were available, five had elevated creatinine phosphokinase (range 174 IU/L–3,422 IU/L), and all had high ferritin levels (range 590 μg/L–4,984 μg/L). Among the four patients (patients 2, 4, 5, and 9) whose follow-up CRP data were available, rising CRP levels were correlated with clinical deterioration (progression of lung infiltrate and aggravation of respiratory distress). A similar correlation was found for serum ferritin levels.

The findings of chest radiographs obtained at admission for the 10 patients ranged from subtle changes, primarily involving reticular interstitial patterns (patients 2 and 10), to focal patchy consolidation (patients 3 to 9), or multilobar patchy consolidations (patient 1). Unilateral opacity occurred predominantly in the upper lung zones. Rapid progression of the lung infiltrate was found in six patients (patients 1–5 and 9). Pleural effusion was found in one patient (patient 1) when he underwent a lung biopsy. Pneumomediastinum developed in one patient (patient 3) 2 days before he underwent endotracheal intubation.

All but patient 1 received ribavirin treatment (Figure 2). Eight patients also received intravenous corticosteroid simultaneously or 1–6 days after ribavirin treatment was initiated, when the patients had persistent fever and worsening lung opacities. The clinical conditions of patients 2 and 3 continuously deteriorated despite the use of ribavirin and corticosteroid. Intravenous immunoglobulin

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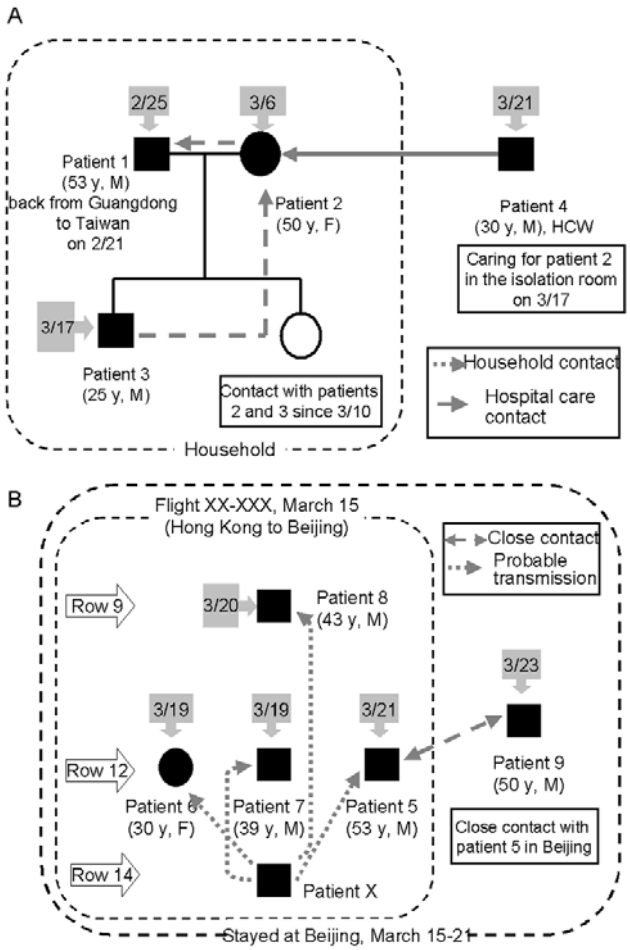

Figure 1. Two clusters involving nine patients with severe acute respiratory syndrome (SARS) who were initially treated at National Taiwan University Hospital. A: One cluster was composed of four patients; two acquired SARS through household contact with an index patient, who had returned from Guangdong Province in China; the fourth was a healthcare worker caring for patient 2. B: The second cluster was composed of four fellow passengers in an airplane (patients 5–8), who sat near a symptomatic patient with SARS (patient X) within the airplane, and one patient (patient 9), who had subsequent close contact with patient 5. Square, male patient; circle, female patient; black, probable case of SARS; blank, healthy person; shading, date of the onset of symptoms; HCW, healthcare worker.

(IVIG) was administrated to treat hemophagocytosis syndrome. Because of the observed effectiveness of IVIG in patient 2, IVIG was administered early (before or simultaneously with corticosteroid) in another four patients (patients 3, 4, 5, 9), whether or not the patient's condition was deteriorating.

Catheter-related infections caused by *Acinetobacter baumannii* and *Candida parapsilosis* developed during the hospital stay of patients l and 9, respectively. *Enterococcus faecium* bacteremia also occurred in patient 9. One of the patients who received ventilator support (patient 2) had concomitant bacterial pneumonia caused by methicillin-resistant *Staphylococcus epidermidis*.

All seven patients (patients 1–5, 9, and 10) had documented infection with SARS-associated coronavirus (CoV) on the basis of positive reverse transcription–polymerase chain reaction (RT-PCR) (polymerase gene) results for their various respiratory and serum samples, positive cultures for SARS-CoV (patients 3, 4, and 9), increased immunoglobulin (Ig) G levels (patients 1–5, and 9), or all of the above (7). The genomes of the three SARS-CoV isolates all were 29,729 nt in length. The sequence of the genome of SARS-CoV from patient 3 (TW1) (GenBank accession no. AY291451) (7) was identical to that of the isolate from patient 4 (TW2). Two nucleotide differences, one in an open reading frame (position 3,165) and one in spike glycopeptide (position 26,477) were found between TW1 and the isolate from patient 9 (TW3).

Histologic examination of the lung biopsy specimen, which was performed on patient 1 on day 18 after onset of illness, showed diffuse interstitial pneumonitis (Figure

Table. Clinical features and laboratory findings of 10 patients with severe acute respiratory syndrome in Taiwan

Clinical and laboratory findings	No. (%) of patients (N = 10)
Clinical features	
Fever (≥38°C)	10 (100)
Dry cough	10 (100)
Loose stool or diarrhea	8 (80)
Myalgia	7 (70)
Malaise	7 (70)
Chills and rigor	6 (60)
Headache	4 (40)
Sore throat	1 (10)
Dyspnea	1 (10)
Abdominal pain	1 (10)
Rhinorrhea	0
Laboratory findings	
Leukopenia (<4 x 10^9/L)	4 (40)
Leukocytosis (>10 x 10^9/L)	1 (10)
Anemia (hemoglobin <12 g/dL)	1 (10)
Lymphocytopenia (<1.5 x 10^9/L)	9 (90)
Thrombocytopenia (<130 x 10^9/L)	5 (50)
Elevated aspartate aminotransferase (AST) (>32 IU/L)	7 (70)
Elevated alanine aminotransferase (ALT) (>40 IU/L)	6 (60)
Elevated lactate dehydrogenase (>460 (IU/L)	7 (78)[a]
Elevated creatinine phosphokinase (>160 IU/L)	5 (71)[b]
Hyponatremia (<134 mmol/L)	5 (50)
Creatinine (>106 mmol/L)	0 (0)
C-reactive protein (>8 mg/L)	
Ferritin (M >377 µg/L; F>151 µg/L)	7 (100)[b]
Oxygen saturation (<95%)	2 (20)

[a]Data were available for nine patients; M, male; F, female.
[b]Data were available for seven patients.

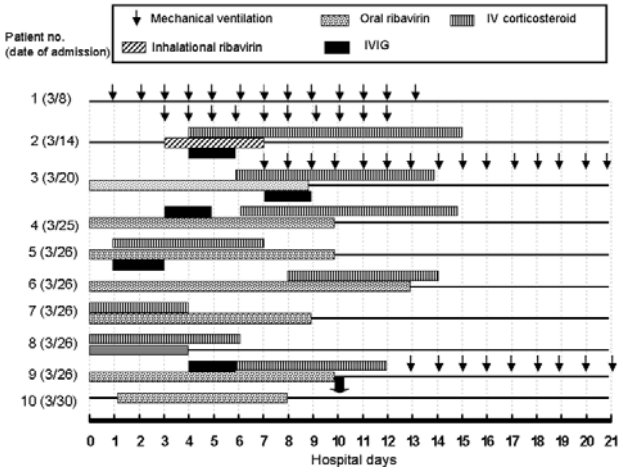

Figure 2. Timeline of therapeutic modalities used in 10 patients with severe acute respiratory syndrome (SARS). IVIG, intravenous immunoglobulin (1 g/kg/d for 2 days). IV corticosteroid, intravenous methylprednisolone (500 mg every day for 3 days, followed by 40 mg every 8 h in patient 9 or 40 mg every 8 h in other patients for 4 to 5 days and then tapered for 1 to 2 weeks). The dosage of oral ribavirin was 2,000 mg initially, then 600 mg every 12 h (or 2,000 mg every day) for 10 days and 0.8 mg/kg every day for inhalational use. Thick arrow, date of discharge.

3A). In addition, syncytial giant cells were scattered in the areas of organizing pneumonia (Figure 3B). No definite intracytoplasmic or intranuclear inclusion was seen, however. Immunohistochemical study showed that the inflammatory cells infiltrating in the interstitia were predominantly CD68 (Dako, Santa Barbara, CA; 100x) positive histiocytes (Figure 3C) and some CD3 (Dako, 100x) positive T lymphocytes. No CD20 (Dako, 100x) positive B lymphocyte or CD56 (Novocastra Laboratory, Tyne, UK; 100x) positive NK cell was found. Pancytopenia developed within 1 to 2 days after ribavirin therapy was initiated and coincidentally when the lung lesions in two patients (patients 2 and 7) were deteriorating and the patients had persistent fever and elevated levels of aspartate aminotransferase and alanine aminotransferase. Bone marrow studies of these two patients disclosed hemophagocytosis (Figure 3D) compatible with a diagnosis of hemophagocytosis syndrome. Results of the RT-PCR assay for these two biopsy specimens were negative.

Serum samples from the seven patients (patients 1–5, 9, and 10), collected in both the acute stage (3 to 4 days before the pulmonary lesion deteriorated) and the convalescent stage (4–6 days after the fever subsided), were subjected to cytokine analysis. Serum levels of interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor α (TNF-α) were determined by using an immunometric assay (IMMULITE, Diagnostic Products Corporation, Los Angeles, CA). All of the serum samples were negative for IL-1β, except for those collected 2–3 days after admission from two patients who had mildly elevated values (patients 5 and 10) (6.8 μg/L and 7.7 μg/L, respectively; reference levels, <5 μg/L). In general, IL-6 and TNF-α levels were high in the acute stage and declined in the convalescent stage (Figure 4). The mean values of TNF-α (reference levels <8.1 pg/mL), IL-6 (reference levels <9.7 pg/mL), and IL-8 (reference levels <62 pg/mL) in the acute stage were 8.83 pg/mL, 27.52 pg/mL, and 266.10 pg/mL, respectively; in the convalescent stage, they were 2.13 pg/mL, 5.42 pg/mL, and 157.15 pg/mL, respectively.

Patient 1 did not receive corticosteroid or IVIG treatment. This patient's levels of TNF-α and IL-6 were high in the early stages of disease and decreased thereafter. The levels of IL-8 in the acute stage in three patients (patients 3, 4, and 9) were high when their clinical condition deteriorated. Although patient 10 lacked an antibody response to SARS-CoV 24 days after illness onset, the changes in IL-6, IL-8, and TNF-α were similar to those of the other six patients.

Conclusions

This study had four important findings. First, there was a high incidence of loose stools or diarrhea in these patients shortly after the onset of febrile episodes and before respiratory symptoms developed. These gastrointestinal manifestations suggest the possibility of viral involvement in the gastrointestinal tract. Although stool cultures from patients 2 and 5 did not grow SARS-CoV, and RT-PCR results for SARS-CoV in these samples were also negative, a previous report clearly demonstrated

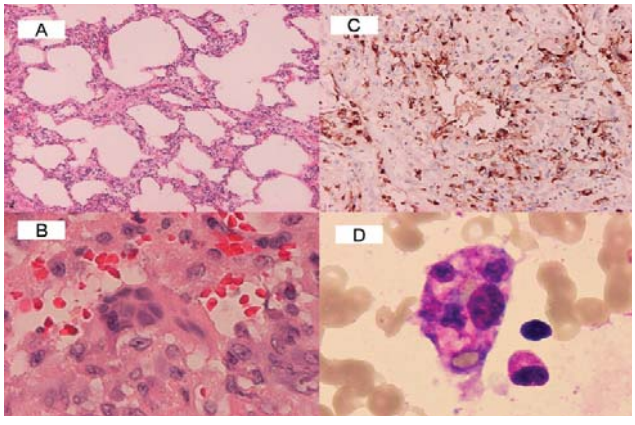

Figure 3. Histopathologic findings of lung biopsy (patient 1) and bone marrow biopsy (patient 2) in two patients with severe acute respiratory syndrome. A: diffuse interstitial pneumonitis with mononuclear cell infiltrating in the mildly thickened alveolar septum (hematoxylin and eosin stain, x100). B: desquamated, multinucleated syncytial giant cell without cytoplasmic inclusion (hematoxylin and eosin stain, x400). C: abundant CD68-positive histiocytes (x100). D: a macrophage ingested with erythrocytes, leukocytes, and platelets (Liu's stain, x1,000).

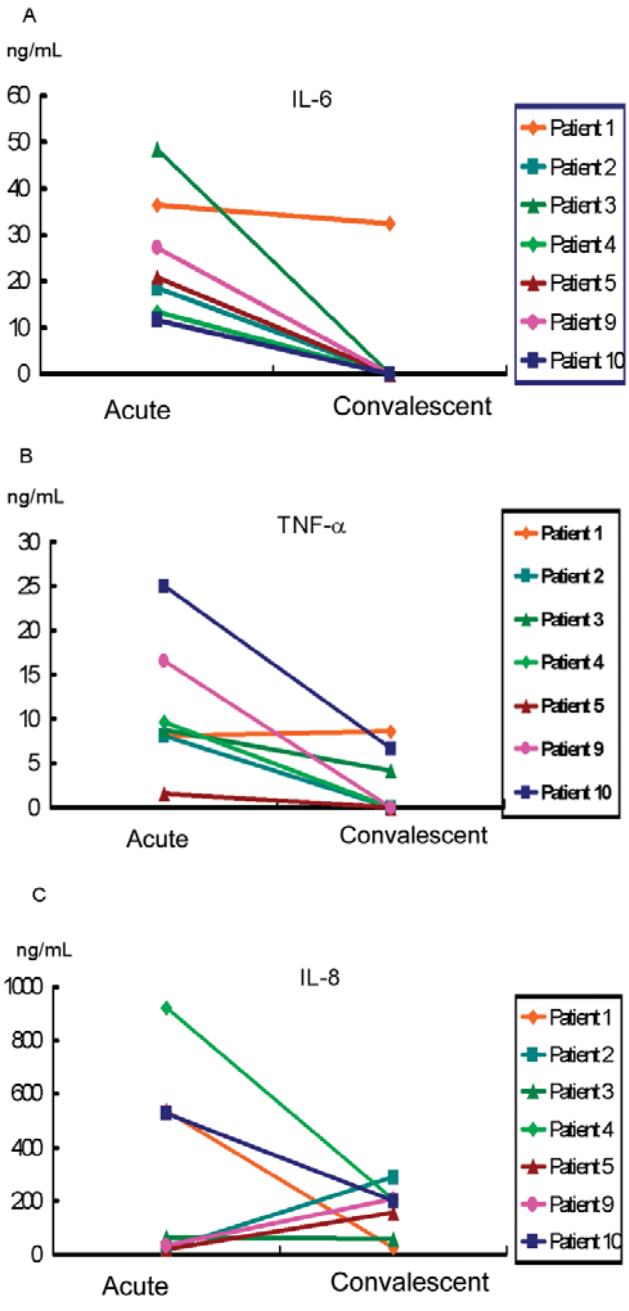

Figure 4. Responses of cytokines during the acute and convalescent stages in seven patients with severe acute respiratory syndrome. A: interleukin (IL)-6 levels; B: tumor necrosis factor (TNF)-α levels; C, IL-8 levels.

SARS-CoV RNA in feces of SARS patients (11).

Second, this study describes, for the first time, finding hemophagocytosis in SARS patients. Bone marrow study should be performed whenever progression of pancytopenia is evident. Moreover, the finding of progression of lung lesions along with pneumomediastinum before endotracheal intubation, which occurred in patient 3, has not been previously reported. This condition might have resulted from extensive alveolar damage. Pleural effusion, as seen in patient 1, has also rarely been previously reported (1–9).

Third, clustering of cases among passengers sitting near a SARS patient during a flight is interesting and important. Due to the closeness of the time of onset of illness in the four patients, a common source is likely and suggests the possible transmission of SARS during the flight.

Finally, levels of certain proinflammatory cytokines, such as IL-6, IL-8, and TNF-α, increased markedly in the acute stage ("cytokine storm") and decreased in the convalescent stage of illness. The data were correlated with the pathologic findings of prominent infiltration of the histiocytes ($HLA\text{-}DR^{+}CD68^{+}$ cells) in the lung and also with the strikingly increased levels of serum CRP and ferritin in the acute stage (12). The levels of cytokines, CRP, and ferritin returned to normal after the patient showed clinical improvement with adequate immunosuppressive treatment. Nevertheless, the immunopathologic changes of cytokines need further investigation because of concurrent bacterial coinfections and the simultaneous use of immunomodulatory agents in our patients (12,13).

Although all of our patients were admitted to negative-pressure isolation rooms that were operated according to strict infection control measures, various nosocomial infections developed in four patients who received mechanical ventilation as well as broad-spectrum antimicrobial agents. Exogenous acquisition of these multidrug-resistant bacteria or *Candida* species from the hospital environment or endogenous selection or induction of resistant organisms under the strong antimicrobial pressure may have both contributed to this phenomenon.

The genetic differences among isolates involved in different nosocomial epidemics need to be compared for efficacious control and prevention of resurgence of this disease (14,15). Previous study indicated that analysis of spike glycoprotein sequences SARS-CoV was useful as a molecular epidemiologic tool (14). Differences of only 2 nt were found between the two SARS-CoV isolates (TW1 and TW3) recovered from patients in the two clusters of infection (7). These findings suggest that these two isolates were closely related and most likely originated from China.

In this study, IVIG was administered early in infection in four patients, whose pulmonary status apparently worsened before IVIG treatment was begun on the basis of its perceived efficacy in another patient. However, each of these patients also received corticosteroid at the same time or soon after, and other factors (delay in start of therapy or different inocula of virus) were possibly associated with the more severe outcome, making it impossible to make a conclusive statement on the role of IVIG on SARS management on the basis of these data alone.

In summary, we described a possible transmission of SARS in an airplane, the occurrence of hemophagocytosis in this newly identified viral infection, and the correlation between serum cytokine levels and clinical course of SARS patients. The lack of a control group in this study makes it impossible to reach any definite conclusions about pathogenesis of illness or efficacy of different treatment modalities.

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B-Virus and Free-Ranging Macaques, Puerto Rico

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In Puerto Rico, risk for transmission of B-virus from free-ranging rhesus monkeys to humans has become a serious challenge. An incident with an injured rhesus monkey, seropositive for B-virus, resulted in inappropriate administration of antiviral postexposure prophylaxis. This incident underscores the importance of education about risks associated with interactions between humans and nonhuman primates.

Two species of introduced nonhuman primates currently thrive on the Commonwealth of Puerto Rico: rhesus macaques (*Macaca mulatta*) and patas monkeys (*Erythrocebus patas*) (1). Although most of the monkeys live in groups in the southwest region of Puerto Rico, recent events might indicate that the primates have spread to the rest of the island, including urban areas.

Both species originated from the La Parguera Primate Facility, which was administered by the Caribbean Primate Research Center of the University of Puerto Rico's Medical Sciences Campus from 1961 until 1982 (2). Primates were introduced onto two peninsulas, Isla Cueva and Isla Guayacan, off the southwest coast of Puerto Rico, near Guánica (Figure). In 1974, the Center, through a contract with the Food and Drug Administration, began to increase the number of breeding female rhesus monkeys to supply animals for the Sabin Poliomyelitis Virus Vaccine Program. Throughout the 1970s, the rhesus colony numbers were increased to >1,000 (3,4). Patas monkeys were introduced to the peninsulas between 1971 and 1981. During this time, an unknown number of monkeys of both species escaped into the regions of Sierra Bermeja, Lajas, Cabo Rojo, and San German. La Parguera Primate Facility ceased operating in 1982, and the monkeys were removed from the facility. However, during the last 20 years, the escapees and their progeny have continued to cause problems in the area, plaguing farmers and concerning public health and environmental officials. Recently, an automobile in an urban area near San Juan (approximately 100 km from La Parguera) hit an adult rhesus monkey. During the incident, a number of emergency personnel were exposed to the monkey's body fluids. The monkey subsequently tested positive for antibodies to B-virus (*Cercopithecine herpesvirus 1)*.

The Study

B-virus is an alphaherpesvirus enzootic among primates of the genus *Macaca* (e.g., rhesus, cynomolgus, pigtailed). First documented in 1932, this virus has received considerable attention because rhesus macaques are a species commonly used in research. Data indicate that 74%–100% of adult rhesus monkeys are seropositive for the virus (5,6). In 1997, a survey of 57 rhesus monkeys in southwest Puerto Rico found that 41 (72%) had serologic evidence of B-virus infection (unpub. data).

Similar to herpes simplex infection in humans, B-virus infection might be asymptomatic or associated with mild lesions or conjunctivitis in macaques (7). Whereas the effects of the virus are mild in macaque hosts, it causes a serious and frequently fatal disease in other primates, including humans. Once transmitted to a human, B-virus infection has a nearly 80% case-fatality rate. To date, <50 human infections have been reported in the literature since infection was first recognized in 1932, despite the multitude of exposures occurring primarily in laboratory environments (7,8). A survey of laboratory workers indicated that asymptomatic seroconversion is unlikely (9). Most transmissions have occurred through monkey bites or scratches, but cases have been documented from needlestick injuries or other laboratory-related accidents, cage scratches, or mucous membrane exposure to monkey body fluids. One isolated case of human-to-human transmission occurred after a woman applied ointment to her husband's infected wound (10). B-virus infection should be recognized early and antiviral prophylaxis given promptly. Early signs of infection in humans include influenzalike symptoms, such as headache, nausea, vomiting, and muscle

Figure. Map of the Commonwealth of Puerto Rico. Nonhuman primates were originally introduced in the southwestern coast, near Guánica.

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pain. Vesicular herpetic lesions, pain, or itching might arise around the area of exposure. The disease progresses quickly to a fulminating meningoencephalitis. Symptoms include paralysis (often progressive and ascending), numbness, ataxia, respiratory difficulties, urinary retention, altered consciousness, and coma. The few cases that have been treated by the time neurologic symptoms have emerged have had limited success (5).

Like other herpesviruses, latency is a feature of B-virus, and many macaques will harbor the virus in trigeminal and lumbosacral ganglia. Stress will increase the likelihood that the virus will be reactivated and shed. Illness, transport, breeding, or environmental stresses have been reported as factors increasing a macaque's likelihood of shedding the virus and therefore becoming infectious. Typically, a very low percentage (2%–3%) of seropositive monkeys will shed the virus at any given time (5). Risk for transmission, therefore, remains quite low.

In February 2002, a total of 25 emergency personnel were exposed to fluids from an injured adult male rhesus monkey that had been struck by an automobile in Bayamón, Puerto Rico. Most persons (n = 22) reported direct contact with the monkey's blood, and none were bitten or scratched by the monkey. B-virus is not a bloodborne pathogen and is not transmitted by blood. However, the possibility of contact with other body fluids that represent a risk for B-virus transmission (e.g., saliva) could not be ruled out in four persons. All 25 persons were interviewed and evaluated by Puerto Rico's Worker's Compensation Agency and placed on prophylactic antiviral medication. All persons were employees from various municipal agencies responding to the accident. None wore latex gloves or other protective gear, despite the fact that most of the persons (84%) had access to personal protective equipment, including latex gloves, masks, and protective eyewear. Current protocols emphasize that the first few minutes after an injury or exposure are the most important (7). In this case, 1 person (4%) washed the exposed area within 1 to 2 minutes of exposure; 11 (44%) waited >5 minutes, 4 (16%) >30 minutes, and 9 (36%) waited >1 hour to clean the exposed area. Most persons used soap and water for cleansing; whether detergents or disinfectants were used is unknown. All 25 persons indicated that they were unaware of potential health risks involved in handling a primate or a rhesus monkey in particular. All were unaware of B-virus. None had been instructed to use protective equipment when handling a monkey. Subsequently, the rhesus monkey tested positive for antibodies to B-virus with enzyme-linked immunosorbent assays (ELISA). Paired serologic samples, completed at weeks 0 and 5 on all 25 persons, were negative for evidence of human infection by B-virus. The center's Virology Laboratory performed the initial ELISA test for the blood sample from the monkey and also confirmed the positive result by sending aliquots of the sample to two independent laboratories (antibodies against B-virus were confirmed by Western Blot [B Virus Resource Laboratory, Atlanta, GA] and by ELISA [BioReliance Corporation, Rockville, MD]).

Conclusions

For 70 years, considerable effort has been undertaken to understand the epidemiology of B-virus to decrease risk for human exposures in research laboratory settings. In the United States, several exposures have resulted from pet macaques (8); however, most B-virus exposures occur inside the laboratory. Work continues to create specific pathogen-free rhesus colonies for research; in the meantime, laboratory personnel are kept well informed and every attempt is made to avoid transmission. Detailed protocols have been developed outlining recommended procedures for human exposure to macaques. Laboratory personnel depend on well-educated healthcare providers that can provide a prompt and knowledgeable management after potential exposures.

Puerto Rico health officials face a novel challenge: free-ranging rhesus monkeys in contact with a largely unaware public. Although the risk for B-virus transmission might be expected to be relatively low, as the monkeys continue to expand their range and population, further encounters with humans should be expected. Rough estimates suggest 500 macaques might be living in southwest Puerto Rico. Data indicate that rhesus populations typically increase their population by approximately 15% each year provided food is available (11).

In southwest Puerto Rico, crop predation is an ongoing problem. Farmers have implemented various tactics to protect their crops, including electric fences, dogs, and possibly hunting or trapping the macaques. Agricultural damage can be considerable after a group moves through a field. Reports document that persons are trapping monkeys, presumably for illegal sale as exotic pets. During a 1993 census, multiple traps were found set up in the forest (Janis González, unpub. data). This finding is alarming, not only from a legal but also from a public health perspective. Trapping and confinement could potentially stress an animal enough to initiate reactivation of latent virus. Inexperienced handlers are at increased risk for a bite or scratch, and fear of legal implications might prevent a person from reporting or seeking medical attention for such injuries. Healthcare providers inexperienced with primates and associated zoonotic diseases might be largely unaware or unfamiliar with B-virus.

Control of the free-ranging, nonhuman primate population promises to be a challenging task. Other examples of introduced primates in the Caribbean offer little conso-

lation. The island of Desecheo is one example; it is located in the Mona Passage between Puerto Rico and Hispaniola (an island divided between Haiti and the Dominican Republic). In 1966, to study adaptation processes, a total of 57 rhesus macaques were released onto the remote 1.2-km^2 island. The monkeys adapted well, and after nesting bird populations dropped substantially on the island, government officials decided to remove the rhesus population (12). Thirty-six years later, after numerous trapping attempts, rhesus macaques still inhabit the island, a National Wildlife Refuge. Barbados suffers similar problems with an introduced species, the African green monkey (*Cercopithecus aethiops sabaeus*). This species was introduced >300 years ago with the slave trade. A study completed in 1994 indicated that despite trapping and removing more than 10,000 monkeys for 14 years, the population still increased by 4.5%; agricultural damage from the monkeys increased almost 30% during the same time span (13).

Although government agencies and other organizations continue to discuss interventions to address the free-ranging population of monkeys, the Puerto Rico Department of Health has begun the process of public education. Informational bulletins for government employees will stress the importance of using personal protective equipment when handling a nonhuman primate and what to do in case of an exposure to a rhesus monkey. In addition, every emergency room will receive a protocol for evaluation and management of exposed persons.

The persons exposed in the incident in Bayamón suffered a low-risk exposure. While the macaque was seropositive for B-virus, most the persons involved were only aware of exposure to blood; no bites or scratches occurred. Exposure to a macaque's blood does not constitute an exposure to B-virus because viremia is thought to be rare among infected macaques (14). Nonetheless, 25 persons were needlessly exposed to fluids from a rhesus, postexposure cleaning was deficient for most persons, and all received an antiviral drug. Prevention of exposure and the unnecessary use of postexposure prophylaxis can be minimized through educational efforts to promote the use of personal protective equipment among first responders and through a better understanding of what body fluids constitute a risk for transmission of B-virus. Furthermore, the general public needs to be aware of this risk and report close contacts with monkeys to health authorities. Other emerging infections, such as monkeypox, underscore the need to remain vigilant against zoonoses.

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Human Metapneumovirus-associated Atypical Pneumonia and SARS

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Acute pneumonia developed in a previously healthy man during the outbreak of severe acute respiratory syndrome (SARS) in southern China in March 2003. Antibiotic treatment was ineffective, and he died 8 days after illness onset. Human metapneumovirus was isolated from lung tissue. No other pathogen was found. Other etiologic agents should thus be sought in apparent SARS cases when coronavirus infection cannot be confirmed.

Human metapneumovirus (HMPV) is a newly identified member of the family *Paramyxoviridae* (1). HMPV infections have been found in all age groups with a wide spectrum of respiratory tract involvement, including a flulike syndrome, bronchitis, bronchiolitis, and pneumonitis (2–5). We report on a patient who died of acute community-acquired pneumonia, from whom HMPV was the only pathogen identified.

Case Report

The patient was a 40-year-old Chinese man with previously good health. He smoked but did not use alcohol. He lived in Hong Kong and took frequent short business trips to mainland China. While in Shenzhen in early March 2003, a fever and productive cough with blood-stained sputum developed. He was hospitalized in Shenzhen and given intravenous antibiotics (the details of which were not available). He remained febrile with chills and rigors, night sweating, and progressive dyspnea. He returned to Hong Kong and sought treatment at Prince of Wales Hospital 8 days after the onset of illness. His temperature was 37.3°C, heart rate 120 beats per minute, blood pressure 110/70 mm Hg, and oxygen saturation 93% on room air. No skin rash was detected. Air entry over the right lung had decreased slightly. Chest radiograph showed patchy consolidations in the right middle zone and mild infiltrates in the left lower zone. An electrocardiogram indicated sinus tachycardia with no ischemic changes.

He was admitted to an isolation ward and given supplemental oxygen and cefuroxime, 750 mg intravenously every 8 h. Blood tests showed leukocytosis (leukocyte count 14.9 x 10^9/L, reference range [RR] 4.0–10.8 x 10^9/L) with neutrophilia (neutrophil count 13.5 x 10^9/L, 91%; RR 41%–73%) and lymphopenia (lymphocyte count 0.5 x 10^9/L, 4%; RR 19%–47%). The hemoglobin and platelet count and results of renal and liver function tests were unremarkable. Arterial blood gas while the patient was receiving 2 L of oxygen showed compensated metabolic acidosis (pH 7.39, RR 7.35–7.45; actual bicarbonate 15 mmol/L, RR 22–26 mmol/L; pO_2 14.2 KPa, RR 10.0–13.0 KPa).

His condition initially improved with normalization of temperature and heart rate, and a satisfactory level of arterial blood oxygen saturation was maintained until early the next morning, when he reported increasing dyspnea and progressed to shock and severe oxygen desaturation. Before he could be transferred to the intensive care unit, he underwent cardiorespiratory arrest, and aggressive resuscitation attempts were not successful.

Microbiologic Investigations

On admission (8 days after the onset of illness), sputum and blood samples were taken for routine microscopy, bacterial (including *Legionella pneumophilia* and mycobacteria), and fungal culture. Polymerase chain reactions (PCRs) targeting *Chlamydia pneumoniae*, *Chlamydia. psittaci*, and *Mycoplasma pneumoniae* were performed on the sputum sample.

Nasopharyngeal aspirate was used for virologic investigations. Rapid viral antigen detection by immunofluorescence was performed with commercial assays to identify influenza A and B; parainfluenza 1, 2, and 3; respiratory syncytial virus (RSV); and adenovirus (Dako Diagnostics Ltd, Ely, UK, and Chemicon International, Inc., Temecula, CA). SARS-associated coronavirus (SARS-CoV) was detected by reverse transcription (RT)-PCR by using primers COR-1 (sense) 5′ CAC CGT TTC TAC AGG TTA GCT AAC GA 3′, and COR-2 (antisense) 5′ AAA TGT TTA CGC AGG TAA GCG TAA AA 3′ that had been shown to be specific for the novel coronavirus detected from patients with SARS (6). Virus isolation was performed using rhesus monkey kidney (LLC-MK2), human laryngeal carcinoma (HEp-2), Mardin Darby canine kidney (MDCK), human embryonic lung fibroblast, Buffalo green monkey kidney (BGM), and African green monkey kidney (Vero) monolayers. Cell monolayers were examined daily for cytopathic effect. After 14 days of incubation, the growth of influenza A and B, parainfluenza 1, 2, and 3, RSV, and adenovirus was examined by commercial

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monoclonal antibodies with the immunofluorescence technique. In addition, a hemadsorption test was performed for LLC-MK2 and MDCK monolayers. A shell vial culture system, coupled with monoclonal antibody–based detection, was used for the isolation of cytomegalovirus.

A serum sample taken on admission (8 days after the onset of illness) was evaluated for hantavirus and atypical pneumonia serology, including *Legionella pneumophila; M. pneumoniae; C. pneumoniae* and *psittaci*, influenza A and B; parainfluenza 1, 2, 3; RSV; and adenovirus. In addition, an in-house immunofluorescence assay based on SARS-CoV–infected Vero cells was used to detect SARS-CoV antibodies. This assay has been successfully applied to specimens from patients with SARS. Results of all microbiologic investigations were negative.

Postmortem Findings

Postmortem biopsy specimens were taken from the lungs, heart, kidney, spleen, small bowel, and muscle. Pulmonary congestion with edema was noted, but hyaline membranes had not formed (Figure A). Interstitial inflammatory cell infiltration was minimal, and intraalveolar organizing lesions were rarely seen, whereas detached atypical pneumocytes were found (Figure B). Atypical multinucleated pneumocytes were observed, but definite viral inclusion was not apparent (Figure C). Fibrin thrombi were frequently observed in small pulmonary arteries and arterioles (Figure D). However, no such thrombotic event was observed in other organs. Central lobular hemorrhagic necrosis was noted in the liver. No myocarditis was observed. Low-grade acute tubular necrosis was seen in the kidneys. Results of examining other tissues were unremarkable. No coronavirus particles were seen on electron microscopy examination of lung cells. The cell culture results were negative, except that a focal refractile rounding cytopathic effect developed 12 days after the left lung tissue sample was injected onto LLC-MK2 cells. This cytopathic effect progressed slowly to cell detachment, and a similar cytopathic effect was observed on other areas of the cell monolayer over the next few days. The cell culture supernatant was positive by an RT-PCR assay based on primers 5′-GAG TAG GGA TCA TCA AGC A-3′and 5′-GCT TAG CTG RTA TAC AGT GTT-3′, targeting the F-gene of HMPV. The nucleotide sequence of the PCR product were identical to the F-gene fragment of HMPV (GenBank accession no. NC 004148) (1). The cell culture supernatant was passaged to LLC-MK2 cells, and the same cytopathic effect was observed after 8 days of incubation. HMPV particles were shown from the passaged cell culture by electron microscopy. To ascertain the presence of HMPV infection in this patient, the serum sample taken on admission (8 days after the onset of illness) was retrieved for HMPV antibody detection with the immunofluorescence technique, based on LLC-MK2 cells infected by the isolated HMPV. An antibody titer of 1:80 was detected.

Conclusions

HMPV is a recently identified human virus associated with respiratory tract disease. While the full range of clinical features is still being elucidated, several cases of severe respiratory tract infections have been reported.

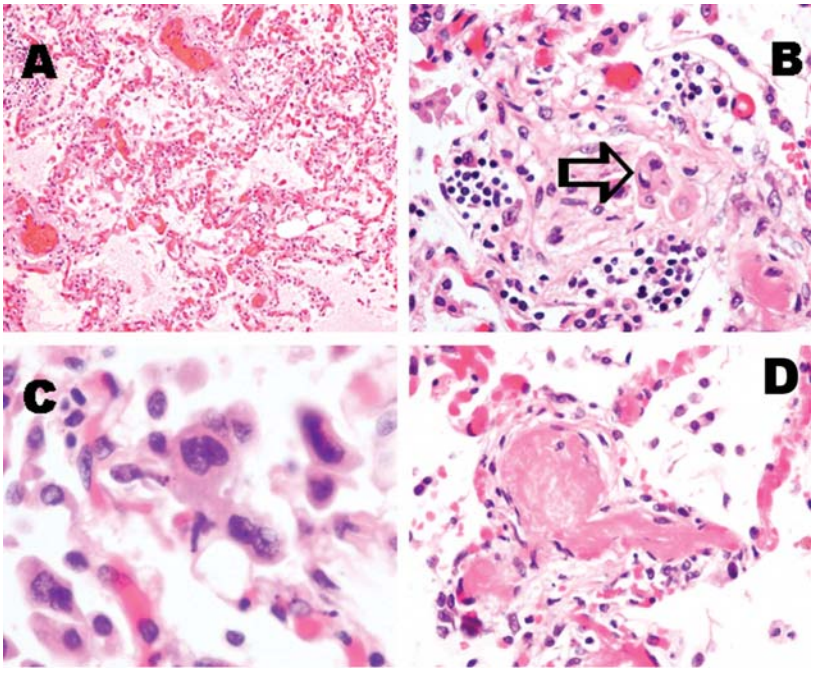

Figure. Pathologic findings of lung tissue sections. A: Pulmonary congestion and edema (H&E stain, original magnification x100). B: A mild degree of interstitial lymphocytic infiltration. Intra-alveolar organizing exudative lesion was occasionally found. Detached atypical pneumocytes indicated by arrow (H&E stain, original magnification x200). C: Atypical multinucleated pneumocytes were occasionally identified. Definite viral inclusion was not apparent (H&E stain, original magnification x400). D: Fibrin thrombi were frequently noted in small pulmonary arteries and arterioles (H&E stain, original magnification x200).

Boivin et al. (2) reported that 3 of 12 infected young children <5 years of age required intensive care, and a child with acute leukemia died subsequently. In that study, one of the six infected adult patients (15–65 years of age) also required intensive care. In the same study, 2 of 10 infected elderly patients (ages >65 years) died; 1 also had leukemia and the other had Alzheimer's disease. However, none of these patients had an autopsy performed. This report is the first in which HMPV was the only pathogen identified from postmortem specimens from a patient with a fatal respiratory tract disease.

Our patient had a history of good health and no evidence to suggest that his death was a result of exacerbation of underlying cardiac or pulmonary conditions. Coinfection has been found in patients with HMPV-associated respiratory tract disease (2) and has been suggested to be a factor influencing clinical outcome (7). In our patient, no evidence of infection with a copathogen could be demonstrated. Although the patient had died before a late convalescent-phase serum sample could be obtained for a confident exclusion of SARS-CoV infection by serologic testing, the nasopharyngeal aspirate collected on admission and the postmortem lung tissue were negative for SARS-CoV by RT-PCR. The postmortem histologic findings on lung tissue of this patient were different from the classic picture of virus-associated interstitial pneumonitis. In addition to the minimal inflammatory cell infiltration, multinucleated giant cells that might represent viral cytopathic effect were only occasionally seen. Rather, the predominant histologic finding was the presence of fibrin thrombi in small pulmonary arteries and arterioles. This thrombotic event was confined to lung tissue and was not found in other tissues. Thrombotic vasculopathy has been observed in infectious diseases caused by HIV, cytomegalovirus, and herpes viruses (8,9). The clinical picture usually resembles thrombotic thrombocytopenic purpura. However, our patient's condition appeared distinct. He showed no serologic or morphologic evidence of other infective agents. He was negative for HIV antibody and did not fulfill the diagnosis of thrombotic thrombocytopenic purpura. The pathogenic event that led to the formation of thrombi was unclear.

Our patient's signs and symptoms, together with his history of travel to southern China, fulfilled the World Health Organization criteria for a probable case of SARS, but no evidence of coronavirus infection could be found. The overall pathologic features of this case were distinct from those in the series of SARS patients that we had examined and that were reported by others (10). In SARS patients, coronavirus particles were seen by electron microscopy in lung cells of most cases, and the lung injuries consistently exhibited features of diffuse alveolar damage. Distinctive airspaces or small airway lesions resembling bronchiolitis obliterans organizing pneumonia were also detected. These SARS-associated pathologic features were not observed in this patient, suggesting a different cause of death. In this patient, HMPV infection was confirmed by virus isolation from lung tissue. Although the possibility of HMPV being a bystander could not be totally excluded, a role of HMPV in severe respiratory tract disease should not be discounted, particularly in SARS patients that do not show evidence of coronavirus infection. HMPV has also been detected in five of the six SARS patients living in Canada; four of them were coinfected with coronavirus (11). The index patient of that cluster had stayed in Hong Kong before the onset of illness. The patient described in this report was unlikely to have a direct link to the outbreak of SARS in our hospital. This patient was admitted around the same time that an outbreak of SARS began in our hospital. That outbreak mainly involved staff working at the Accident and Emergency Department and the index ward where a SARS patient had been treated. The index ward is located on a different floor from where the patient described in this report was isolated. In addition, neither fever nor respiratory illness developed in any of the healthcare workers involved in taking care of this patient in the next 2 weeks, and we were not aware of any spread to his family members.

Although HMPV and SARS-CoV might differ in transmission efficiency, their role and clinical outcome in acute respiratory tract disease need to be distinguished. This distinction is particularly important for analyzing the illness and death associated with the worldwide outbreak of SARS. Patients fulfilling the clinical criteria for a probable case of SARS might be infected by organisms other than coronavirus.

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Q Fever Endocarditis in HIV-Infected Patient

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We describe a case of Q fever endocarditis in an HIV-infected patient. The case was treated successfully with valvular replacement and a combination of doxycycline and hydroxychloroquine. We review the current literature on Q fever endocarditis, with an emphasis on the co-infection of HIV and *Coxiella burnetii*.

Coxiella burnetii is classified in the order Legionellales and is closely related to *Legionella* and *Francisella* spp. (1). This zoonotic agent has been isolated from various birds, mammals, and arthropods and is considered endemic in cattle in some regions of the United States (2,3). Although usually nonpathogenic in animals, outbreaks of *C. burnetii*–induced abortions have been described in goats and sheep. In humans, *C. burnetii* is acquired primarily by inhaling infectious dust (4). The bacteria are able to survive in a sporelike form under harsh environmental conditions and are extremely contagious: a single organism can cause disease (5). Q fever manifests as acute or chronic disease. The acute disease may include an undifferentiated febrile syndrome, pneumonia, or hepatitis. The most common chronic symptom is endocarditis (6). We describe a case of Q fever endocarditis in a patient with HIV infection. This co-infection has never been reported in the United States and rarely has been described elsewhere.

The Study

A 46-year-old man with HIV infection was admitted to an emergency department in August 2002 with sudden onset of chest pain and shortness of breath. The patient was born in Mexico and recalled contact with farm animals and consuming unpasteurized dairy products while raised in the state of Chihuahua. He migrated to United States in 1987 and returned periodically to Mexico, most recently in 1998. While residing in the United States, the patient had no known direct or indirect exposures to ruminants.

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Ten months before admission (November 2001), the patient had been hospitalized with pneumonia and was diagnosed with HIV infection. One month later, the patient was hospitalized because of *Candida* esophagitis and thrombocytopenia. A bone marrow biopsy obtained during that hospitalization showed adequate megakaryocytes, mild megaloblastic changes in erythroid precursors, and adequate iron stores. Special stains and cultures for acid-fast bacilli and fungi were negative, as were blood cultures for *Mycobacterium avium* complex. During that admission a diastolic murmur was noted, and an echocardiogram showed severe aortic insufficiency with a thickened aortic valve. The patient was discharged on antiretroviral therapy with lamivudine, stavudine, and nelfinavir, diuretics and ACE inhibitors.

Seven weeks before the first hospitalization in November 2001, the patient was admitted with headache, neck rigidity, and chills. Computed tomography scans of the head and neck and a lumbar puncture showed no abnormalities. The symptoms resolved with the use of empiric intravenous vancomycin and ceftriaxone. Because of persistent thrombocytopenia, a second bone marrow biopsy was performed in February 2002. Granulomas were identified in the biopsy, although special stains for acid-fast bacilli and fungi were negative. Treatment with clarithromycin, ethambutol, and rifabutin for presumed *M. avium* complex was initiated. and the patient was discharged.

On examination, pulse was 101 beats per minute, temperature was 37.0°C, respiratory rate was 22 beats per minute, and blood pressure was 113/42 mm Hg. Mild temporal wasting, absence of oral thrush, poor dentition, positive jugular venous distention, bilateral crackles in the lungs, a decrescendo diastolic murmur in the left sternal border, "water hammer" pulses, and clubbing of the digits were found. Serum sodium level was 129 mEq/L; creatinine 1.4 mg/dL; albumin 3.2 mg/dL; normal liver function tests; leukocyte count 8,800/μL, with 90.8% neutrophils and 7.3% lymphocytes and hemoglobin 8.2 g/dL and platelets 72,000/μL. Antibodies to nuclei and smooth muscle were detected at titers of 1:40 and 1:160, respectively. $CD4^+$ lymphocyte count was 82 cells/mm^3 and viral load 2,838 copies/mm^3. Chest x-ray showed cardiomegaly and congestive heart failure. An electrocardiogram showed a left anterior hemiblock and a first-degree atrioventricular block. A transthoracic echocardiogram showed a large vegetation on the aortic valve and severe aortic insufficiency. Other findings included left ventricular dysfunction, a structurally normal mitral valve with a small vegetation on the atrial surface of the anterior leaflet, and mild mitral regurgitation. Empiric treatment for bacterial endocarditis was initiated with oxacillin, gentamicin, and ampicillin. Because of severe aortic insufficiency, the patient's aortic

valve was surgically resected and showed a severely fenestrated, tri-leaflet valve with bulky, white, irregular vegetations. The mitral valve was free of vegetations, but was perforated and required repair with an autologous pericardial patch. Initial histopathologic evaluation of the excised aortic valve reported scant inflammation and extensive calcification and hyalinization. The patient did well postoperatively and was discharged after 4 weeks of intravenous antimicrobial therapy. Blood cultures obtained before antimicrobial drugs were administered failed to grow routine aerobic or anaerobic bacteria, mycobacteria, or fungi.

After discharge, serum samples tested by an indirect immunofluorescence antibody (IFA) assay showed immunoglobulin (Ig) G antibodies reactive with phase I and II antigens of *C. burnetii* at reciprocal titers of 16,384 and 16,384, respectively. The resected valve was sent to the Centers for Disease Control and Prevention. Histopathologic evaluation showed calcification and hyalinization and foci of prominent mononuclear infiltrates with occasional multinucleated giant cells. An immunohistochemical stain for *C. burnetii* using a polyclonal mouse primary antibody reactive with *C. burnetii* was applied to sections of valve tissue. Abundant intracellular staining of *Coxiella* antigens was identified in foamy macrophages in areas of inflammation and calcification (Figure). The patient was started on doxycycline 100 mg per os each day and hydroxychloroquine 400 mg per os each day. During a follow-up visit 4 months after hospitalization, the patient was clinically asymptomatic, platelet count was 146/μL, albumin was 4.2 mg/dL, and no antinuclear antibodies were detected. Follow-up antibody titers against phase I and II antigens of *Coxiella* were both 4,096.

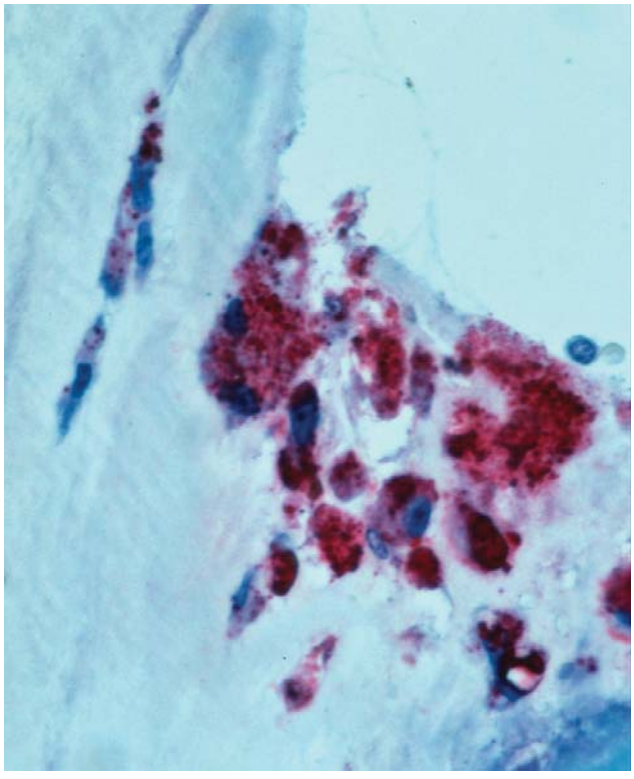

Figure. Immunohistochemical localization of *Coxiella burnetii* antigens in the aortic valve of a patient co-infected with HIV. Intact bacteria and fragment antigens are identified predominantly within macrophages in the fibrosed and calcified valve tissue. (Immunoalkaline phosphatase stain with naphthol phosphate fast-red substrate and hematoxylin counterstain, original magnification x100).

Conclusions

Q fever has a worldwide distribution (7) and, in the United States, was made nationally notifiable in 2000. The disease is not reportable in Mexico, our patient's country of origin, and only one case report of Q fever endocarditis has been published from that country (8).

Pneumonia developed in our patient several months before endocarditis was diagnosed; however, the role of *C. burnetii* as the etiologic agent of his prior pulmonary disease is not known. A syndrome characterized by absence of fever; insidious aortic valvular dysfunction, leading to congestive heart failure; anemia; thrombocytopenia; and autoantibodies subsequently developed in our patient. Routine blood cultures were negative. Culture-negative endocarditis is implicated in 6% of cases of infective endocarditis in HIV-infected patients. Some of the pathogens identified as the cause of culture-negative endocarditis in this patient cohort include *Bartonella*, *Coxiella*, and *Arcanobacterium*. However, because diagnostic assays for Q fever are attempted infrequently, the number of culture-negative endocarditis cases caused by *C. burnetii* is unknown (9–11).

Although infections with certain intracellular organisms (e.g., *Mycobacterium*, *Salmonella*, and *Leishmania* spp.) are more frequent and have more severe signs and symptoms in HIV-infected patients, the prevalence and severity of Q fever in persons infected with HIV compared with the general population remain controversial. Raoult et al. (12) found a threefold increase in prevalence of Q fever serologic findings among HIV-infected patients in Marseille, France (2.4% vs. 0.8%), and determined an annual incidence of 2.7 per 100,000 in the general population and 33 per 100,000 in HIV-seropositive patients (4). However, other seroprevalence studies failed to confirm an increase of Q fever in HIV-seropositive patients (13–15). A study from Italy described two outbreaks of Q fever in a residential facility for drug abusers. In the first epidemic, the Q fever attack rate was 45.9% for HIV-seropositive persons compared with 25.1% for HIV-seronegative persons. However, during the second outbreak 1 year later, no

significant association with HIV coinfection or $CD4^+$ cell count existed, and no significant differences occurred in the levels of antibodies or the clinical signs and symptoms between these patient cohorts in either outbreak (16).

Even less information is available about Q fever endocarditis in persons infected with HIV. *C. burnetii* is controlled by a nonsterile immunity and perhaps is never cleared from an infected patient (17). Because *C. burnetii* is an intracellular pathogen, Q fever endocarditis might be expected to occur with greater frequency in HIV-infected patients than in the general population; however, most cases of chronic Q fever occurring in immunosuppressed patients have been reported among persons with cancer (6) and only rarely among HIV-infected persons (6,12).

Q fever endocarditis was suspected in our patient on the basis of his clinical manifestations, history of exposure to farm animals, and absence of bacterial growth in routine blood cultures. Conventional blood cultures for *C. burnetii* are characteristically negative, but use of shell vial cell culture assay techniques are more sensitive and less hazardous than conventional blood cultures (18).

The diagnosis in our patient was confirmed by serologic and immunohistochemical methods. Chronic Q fever can be diagnosed by detection of high anti-phase I IgG antibody titers by IFA, complement fixation, or enzyme immunoassay (19). IFA is considered the serologic standard criterion and was the test used in our patient. Nonspecific, low-dilution seropositivity for *Coxiella* has been reported in HIV-seropositive persons by IFA (12), but our patient had very high titers compatible with *Coxiella* infection. Also, some patients with *C. burnetii* have been found to have false-positive results by HIV enzyme-linked immunosorbent assay (20,21). This finding was not the case in our patient, who had a confirmatory Western blot for HIV and a low $CD4^+$ cell count.

Echocardiography showed a large vegetation in our patient. This finding is relatively unusual in patients with Q fever endocarditis, and transthoracic echocardiogram rarely demonstrates vegetation (6). Because of the difficulty of diagnosing Q fever endocarditis with the current Duke's criteria, particularly when blood cultures are negative and vegetation is absent, a modification has been suggested so that a single positive blood culture or a high anti-phase I antibody titer is considered diagnostic (22).

A combination of doxycycline and hydroxychloroquine was given to our patient, who is currently clinically well. Although no data specifically describe the treatment of Q fever endocarditis in HIV-infected patients, the combination of both antimicrobial drugs appears to be an effective therapeutic regimen for this disease. Hydroxychloroquine increases the pH of phagolysosomes, enhancing the activity of doxycycline against *C. burnetii*. This combination of drugs can reduce the duration of treatment from 3–4 years to 18 months (23), although some authorities recommend that treatment be continued indefinitely.

Q fever endocarditis is a potentially severe infection, with a case-fatality ratio of approximately 24% in historical case series (6). Earlier diagnosis and newer treatment combinations may improve survival and decrease rates of recurrence. Further studies are required to evaluate the long-term prognosis of Q fever endocarditis in patients with HIV. Q fever is infrequently diagnosed in persons with endocarditis because of its relative rarity and because it is seldom considered in the differential diagnosis. However, it should be considered in all patients with culture-negative endocarditis, particularly those with appropriate risk factors that include past or current exposure to livestock.

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Bovine Necrotic Vulvovaginitis Associated with *Porphyromonas levii*

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An outbreak of bovine necrotic vulvovaginitis associated with *Porphyromonas levii*, an emerging animal and human pathogen, affected 32 cows on a dairy farm in the northeast of Israel. Five animals had to be culled. This report appears to be the first that associates *P. levii* with bovine necrotic vulvovagnitis.

Porphyromonas levii is an emerging pathogen of human and veterinary importance. *P. levii*–like microorganisms were isolated from various human and animal infections (1–5). Investigation of *P. levii* virulence factors (6,7) indicated that this microorganism is able to synthesize an anti-IgG_2 protease (8) and to reduce macrophage chemotaxis, phagocytosis, and oxidative burst. These activities were obviated by the presence of anti-*P. levii* serum or immunoglobulin (Ig) G, indicating that acquired immunity might be important in preventing infection (4).

In Israel, for the last several years, the tendency has been to combine dairy herds into large holdings, for economic reasons. Thus, large groups of cattle are often moved and introduced into new environments that might substantially differ from those where they originated. During the end of 2000 and the beginning of 2001, outbreaks of bovine necrotic vulvovaginitis (BNVV), lasting about 4 months, were observed in three dairy herds in northeast Israel, several months after the introduction of new stock. Heifers and transferred cows were affected more frequently than multiparous and local cows, respectively.

Towards the end of 2001, two similar outbreaks of BNVV were reported, one of them in a previously unaffected herd. The heifers of this herd were transferred as calves to be raised on another farm and returned before calving. BNVV was not observed on the hosting farm. A third outbreak began in January 2002 on a farm that had been affected during the same season 1 year previously. This outbreak was followed from its onset to its conclusion and is described in this report.

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Outbreak

The herd comprised approximately 550 dairy cows of the Israeli-Holstein breed raised in a zero-grazing management system. Heifers were transferred at the age of 2 months to another farm and returned pregnant 14 months later. All the heifers were kept together in one group. All the cows and heifers were observed at least twice daily by the herd's personnel and at least twice weekly by the attending veterinarian.

Cases of BNVV were observed during January through March 2002. Thirty-nine heifers calved during this period and were included in this study. Clinical signs of BNVV developed in 32 of the 39 heifers. Other age groups and heifers before calving were not affected. Clinical signs appeared during the first week after calving. The prodromic phase of the lesions was characterized primarily by erythema. Subsequently, the lesions progressed to hemorrhagic necrosis (Figure 1). Affected cows were not isolated from the rest of the herd. Treatment consisted of vaginal rinses with potassium permanganate or H_2O_2 daily from calving for as long as clinical symptoms persisted (9). Local antimicrobial therapy was not attempted since it was ineffective during the outbreaks on the other farms. A penicillin/streptomycin combination was given to 12 cows with temperatures >39.5°C, to provide broad-spectrum protection, prevent sepsis, and limit economic losses (metronidazole is the drug of choice to treat infections

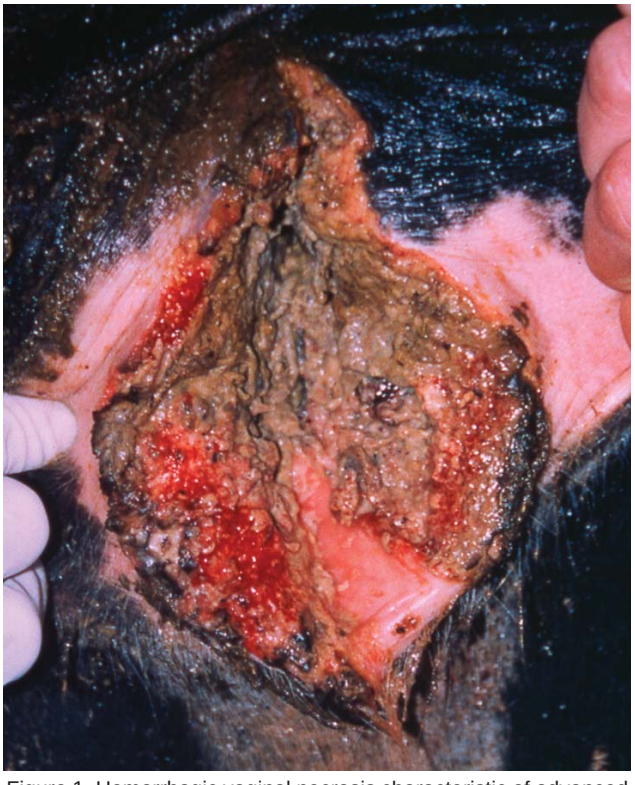

Figure 1. Hemorrhagic vaginal necrosis characteristic of advanced bovine necrotic vulvovaginitis.

caused by anaerobic bacteria, but its use is prohibited in Israel). Five of these cows developed metritis and peritonitis and had to be slaughtered, whereas the temperature of the remaining seven cows returned to normal. The systemic treatment had no clinical or bacteriologic effect on the vulvovaginal lesions. No difference between convalescence periods of cows treated with potassium permanganate or H_2O_2 was observed. Systemic antimicrobial therapy had no discernible effect on the vulvovaginal lesions. Eighteen cows recovered within 4 weeks after onset of infection; in 9, a more chronic infection, characterized by a mucopurulent vaginal discharge lasting up to 10 weeks, developed.

Laboratory diagnosis was performed on samples taken at various stages of the outbreak. The perineum of each cow was washed and disinfected with 70% alcohol before sampling. Vaginal swabs for bacteriology were taken weekly. The swabs were kept in Amies Transport Medium (Copan, Italy) and processed within 5 hours from sampling. Swabs for mycoplasma and ureaplasma were taken in Mycoplasmal Transport Medium and Hayflick's Medium, respectively (10). Swabs for virologic examination were placed in Eagle's medium supplemented with antimicrobials and fetal calf serum and chilled. After vortexing, the medium was used for polymerase chain reaction (PCR) assays and virus isolation. A total of 136 samples from 39 cows were examined bacteriologically. From these, 82 samples from 38 cows were examined virologically as well. Vaginal biopsies were suspended directly in 10% formalin and stained with hematoxylin-eosin in the laboratory.

Bacteriologic examination included aerobic, microaerophilic, and anaerobic cultures, and resulting microorganisms were identified by standard methods (10). Samples were examined for *Chlamydia* by direct immunofluorescence (Cellab, Australia) and for *Coxiella burnetti* by the Stamp (11) staining method. The only microorganisms cultured consistently from affected cows but not from healthy ones were pigmented, gram-negative, non–spore-forming, anaerobic rods. Autosatellitism was observed in several instances. The number of pigmented colonies was directly related to the severity of the vulvovaginal lesions, and they were not cultured from cows after their recovery.

The pigmented bacteria were weakly saccharolytic; negative for indole, catalase, esculin hydrolysis, α-fucosidase, and α-galactosidase; and positive for β-galactosidase and N-acetyl-β-glucosaminidase (as determined by the API rapid ID 32A kit, [bioMérieux, France]). Identification of pigmented isolates as *P. levii* was done according to the Manual of Clinical Microbiology (12). Since susceptibility to vancomycin distinguishes *Porphyromonas* spp. from other gram-negative anaerobic rods (12) the MIC of the isolates to this antibiotic was determined by the Etest method (AB Biodisk, Sweden) and was found to be 3 μg/mL, indicating susceptibility. Other potentially pathogenic bacteria isolated occasionally included *Arcanobacterium pyogenes*, α-hemolytic streptococci, and several *Enterobacteriaceae*, but they could not be correlated with either the clinical signs or the presence of *P. levii* or bovine herpesvirus 4 (BoHV-4).

For the virologic examination, samples were added to confluent monolayers of Madin-Darby bovine kidney cells, observed daily for cytopathic effect, and passaged every 7 days. Samples were considered negative if no cytopathic effect appeared within 3 weeklong passages. Isolates of BoHV-4 were identified by PCR (L. Koznetzova, et al., unpub. data). BoHV-4 was detected in samples from 27 cows with BNVV and from two cows in which the syndrome did not develop. It was not found in four healthy and five infected cows. Homology with the bB gene of BoHV-4 was >98%. BoHV-1 was isolated from 11 samples, alone or in conjunction with BoHV-4.

Association of the bacteriologic or virologic findings and the clinical status of the cows was assessed statistically by the Mantel-Haenszel test (Statistix, Analytical Software, Tallahassee, FL). Data used for the Mantel-Haenszel test are presented in the Table. The test indicated a significant association between cows with BNVV and the presence of *P. levii*, adjusted for BoHV-4 ($p = 0.0006$). No statistically significant association between BoHV-4, adjusted for *P. levii*, and BNVV was found ($p = 0.2359$). Previous publications (13) also reported difficulties establishing a clear relationship between the presence of BoHV-4 and, among other syndromes, bovine metritis, vaginitis, and abortions.

Uterine biopsies were not taken, as injuring the mucosa in the presence of vaginal lesions could increase the risk for metritis. One cow, diagnosed with metritis and peritonitis, was necropsied. Bacterial colonies were seen in the section of vaginal biopsies, but typical changes of BoHV-4 infection (13) were absent. The single necropsied cow showed peritonitis, resulting from rectal rupture, the cause of which could not be determined. Numerous pseudomembranous ulcers, covered with pus, were seen on the vaginal mucosa. The lesions affected the vulva and caudal part of the vagina but not the uterus (Figure 2). Histopathologic examination of the vaginal lesions showed extensive necrosis of the epithelium and severe infiltration with a

Table. Results of bacteriologic and virologic examinations in outbreak of bovine necrotic vulvovaginitis in Israeli-Holstein dairy cows on a farm in the northeast of Israel[a]

Findings	No. cows in which clinical signs present	No. cows in which clinical signs absent
P. levii isolated	32	2
P. levii not isolated	0	5
BoHV-4 found	26	2
BoHV-4 not found	5	5

[a]*P. levii, Porphyromonas levii*; BoHV-4, bovine herpesvirus 4.

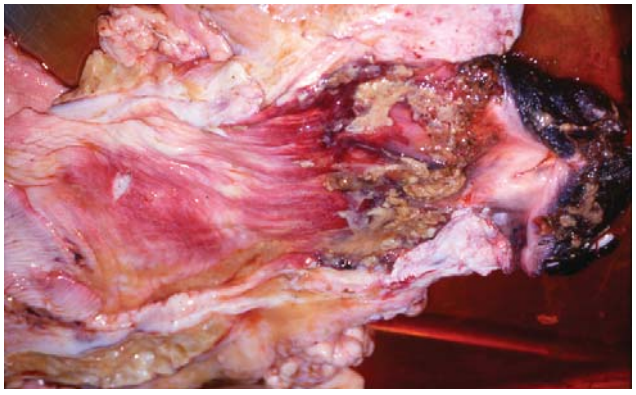

Figure 2: Pathologic lesions of the vagina; note involvement primarily of caudal region.

large number of mostly degenerative neutrophils and foamy macrophages. In the submucosa, vascular proliferation was evident.

Conclusions

Since *P. levii* was isolated from all BNVV cases and very few healthy cows, it likely caused the lesions. Statistical analysis of data corroborates this assumption. All the cases observed during this outbreak occurred in primiparous cows in a restricted geographic area (within a radius of 20 km) during a limited period of time, indicating that one or more risk factors, alone or in conjunction, predisposed cows to infection. Outbreaks were observed after a large number of cattle were introduced, affecting primarily the animals introduced into the host farm. Transportation and social conflict may have acted as stressors (with stress' immunosuppressive effects) (14) and predisposed the introduced cows to infection, with only social conflict affecting the local cattle. An additional risk factor might have been the age and primiparity of the cows. Calving is a well-known stressor (15), especially in primiparous cows (16). Moreover, lesions of the genital tract caused by calving tend to be more severe in primiparous cows, thus making them more prone to infection.

After BNVV was described and its putative etiologic agent was identified, several sporadic cases were diagnosed on other farms, indicating that it might be underdiagnosed, and further studies of the syndrome may be warranted.

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Neurocysticercosis in Oregon, 1995–2000

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The unexpected death of a teenager from neurocysticercosis prompted an investigation of this disease in Oregon. We found 89 hospitalizations, 43 newly diagnosed cases, and 6 deaths from 1995 to 2000. At least five cases occurred in persons who had not traveled or lived outside the United States. Enhanced surveillance for neurocysticercosis is warranted.

Neurocysticercosis, an infection of the central nervous system with the larval form of the pork tapeworm *Taenia solium*, causes substantial illness and death in developing countries. The disease has recently been increasingly recognized as a public health problem in the United States, primarily in southwestern states bordering Mexico (1). In nonborder states, however, neurocysticercosis may be an underrecognized problem.

In September 2000, Oregon's Statewide Child Fatality Review Team reviewed the unexpected death of a 17-year-old girl due to neurocysticercosis; the diagnosis was made postmortem. We describe this case and present the results of the investigation of the epidemiology of neurocysticercosis in Oregon that followed.

Case Report

A previously healthy teenage girl, who had immigrated to Oregon from Mexico as an infant, sought care in January 2000 for progressively severe headaches of several months duration. After three office visits, "tension headaches" were diagnosed, and symptomatic therapy was prescribed. Neuroimaging was not performed. Several days later she was found dead at home in her bed. Autopsy showed no evidence of trauma, and results of a toxicology screening were negative. Examination of the brain showed obstructive hydrocephalus, bilateral uncal herniation, flattening of the cerebral gyri, and an intact cysticercus compressing the inferior 4th ventricle (Figure 1).

The Study

We searched the Oregon Hospital Discharge Database to identify Oregon hospitals that had discharged patients with the ICD-9-CM code for neurocysticercosis (123.1) from January 1995 through December 2000 and requested the medical records of these patients. Available medical records were abstracted by using a standardized data collection instrument. We searched the Oregon death certificate database for additional neurocysticercosis deaths during the same period.

A case of neurocysticercosis was defined as any person with a hospital discharge code of 123.1 or death certificate diagnosis of neurocysticercosis during the study period, and a record of imaging studies or pathology reports consistent with neurocysticercosis. An incident case was defined as one for which no reference to any previous diagnosis of neurocysticercosis was found in the medical record. Incidence rates were calculated by using U.S. Census Bureau yearly population estimates for Oregon as the denominator (2,3). Data were analyzed with EpiInfo, version 6.04d (Centers for Disease Control and Prevention, Atlanta, GA).

We found 89 hospital discharges coded for neurocysticercosis during the study period among 18 hospitals in 10 counties. Medical records were made available for 76 (85%) of these hospitalizations. Review of these records

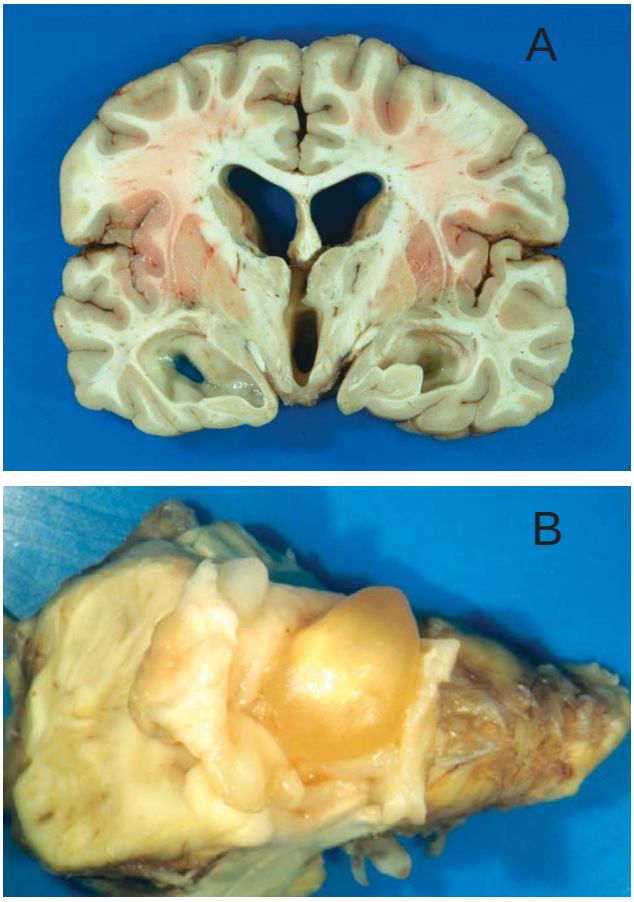

Figure 1. A) Coronal section of brain showing dilation of ventricles, flattening of the cerebral gyri, and uncal herniation. B) Intact cysticercus occupying the 4th ventricle

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confirmed the diagnosis of neurocysticercosis for 59 persons (17 were admitted more than once). Review of death certificates showed one additional nonhospitalized case-patient. Thus, including the death described above, we found 61 persons who met the case definition for neurocysticercosis; 43 (70%) of these were incident cases. Figure 2 shows neurocysticercosis hospital discharges and new diagnoses by year. The annual number of incident cases did not change significantly during the study period. The mean annual incidence rate from 1995 to 2000 was 0.2 per 100,000 general population and 3.1 per 100,000 Hispanic population.

Among the 61 confirmed patients, 40 (66%) were male, and 52 (85%) were Hispanic. The median age at the time of first hospitalization was 24 years (range 2 to 79 years); 13 (21%) were <18 years old. Occupation was recorded for 37 adult patients. Agriculture or other manual labor was the most common job type listed (20/37; 54%); 21% patients were unemployed or disabled; 40% of patients had no health insurance.

The country of birth was recorded for 57 persons. Of these, 41 (72%) were born in Mexico, 10 (18%) in the United States, 3 (5%) in Guatemala, and 1 (2%) each in Korea, Saudi Arabia, and an unspecified African country. Of the 10 neurocysticercosis patients born in the United States, 5 had never traveled outside the United States. Four of these five cases occurred among children (ages 3, 5, 5, and 12 years); their source of exposure to *Taenia* eggs was not documented. The source of exposure for the adult patient was presumed to be a household contact visiting from Mexico. Travel histories of four of the remaining U.S.-born patients included a Caribbean cruise by a retired pig farmer, extensive travel in Madagascar and Tanzania by a student, annual visits to Puerto Rico by an 11-year-old, and 1 week spent in Mexico City by a toddler. No information about travel was documented for one patient, a 3-year-old child.

The median duration of hospitalization was 3 days (range 1–30 days). Admission to an intensive care unit occurred in 21% of hospitalizations. Treatment included craniotomy with cyst removal (16%), ventriculoperitoneal shunting (12%), albendazole (18%), praziquantel (19%), anticonvulsive therapy (63%), and corticosteroids (49%).

Five persons died in addition to the patient described here. Two of these deaths also occurred unexpectedly before diagnosis; both resulted from acute hydrocephalus caused by an obstructing cyst on the 4th ventricle. Three patients who had been previously diagnosed with neurocysticercosis died. The causes of death in these patients were aspiration pneumonia after craniotomy and cyst removal, intracerebral hemorrhage, and hydrocephalus due to recurrent cerebrospinal fluid shunt failure. The median age at time of death was 33 years (range 17–73 years).

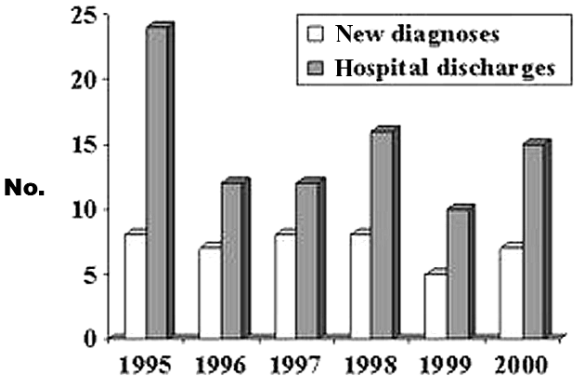

Figure 2. Number of neurocysticercosis hospital discharges and new diagnoses by year; Oregon, January 1995–December 2000.

Conclusions

This study demonstrates that neurocysticercosis causes substantial illness and death among Hispanic populations in Oregon. Many of those affected are young immigrants from Mexico without medical insurance, who are either unemployed or are working in agriculture or other manual labor. At least five cases appear to have been acquired in the United States. Our study did not address where disease transmission occurred for persons born outside of the United States; some of these cases could also have been acquired locally.

Several previous reports have documented the increasing recognition of neurocysticercosis in the United States, but the emphasis has been primarily on disease occurring in southwestern states bordering Mexico (4) and small outbreaks elsewhere (5–7). Previously, the only available information about the occurrence of neurocysticercosis in the Pacific Northwest was a study of seizure patients at 11 university-affiliated, urban emergency departments throughout the United States. In that study, cases were concentrated in southwestern states; four cases were found in Oregon during a 2-year period (8). The study underestimated the incidence of this disease in the Northwest, and perhaps other areas of the United States, by looking for cases only in selected urban emergency departments.

The average annual incidence of neurocysticercosis among Oregon's Hispanic population found in our study is higher than that previously reported in Los Angeles County (1.6/100,000) (9) and in Mexico (0.8/100,000) (10). Low U.S. Census Bureau population estimates due to undercounting of Hispanic migrants could have resulted in a falsely elevated incidence rate. In addition, the higher observed incidence in Oregon may have been a result of greater case ascertainment because hospital discharge data were used rather than physician and laboratory reports. Nevertheless, because of our study design, we probably underestimated the true number of incident cases. Persons

in outpatient clinics and emergency departments in whom neurocysticercosis had been diagnosed (who did not become hospitalized) were not counted in our study. We also did not include in the analysis of cases 15% of hospital discharge diagnoses that we could not confirm by chart review; if these had been confirmed as neurocysticercosis, then the true number of new cases may have been even higher. Although the Hispanic population in Oregon grew by an estimated 67% during the study period (2,3), the number of hospitalizations and new diagnoses did not increase. Whether this finding represents a true decrease in incidence or a shift in diagnosis and management of these cases to the outpatient setting, is unclear.

Neurocysticercosis has not previously been a reportable condition in Oregon, and no public health followup of the patients with locally acquired cases was performed to determine the source of these persons' exposures to *Taenia* eggs. Previous serologic studies (11,12) would suggest that these patients acquired disease from household contact with a *T. solium* carrier. Early identification and public health followup of neurocysticercosis patients may lead to the recognition and treatment of tapeworm carriers, thereby preventing additional cases. However, since excretion of *Taenia* eggs is intermittent, direct parasitologic examination of stools is not a sensitive test (11). Collecting multiple stool samples from asymptomatic persons may also be a challenge. A serologic test for detection of taenaisis has been developed (13), but it is not yet commercially available. Public health providers need simple, inexpensive, and readily available techniques for rapidly identifying *Taenia* carriers in order to conduct optimal followup of cases and prevent additional cases.

The World Health Organization has estimated that more than 2 million persons are infected with adult tapeworms (14). As a result of increasing immigration and foreign travel, *T. solium* will likely continue to emerge as an important pathogen in the United States. Cysticercosis and taeniasis were designated as reportable conditions in Oregon in 2002. We hope that public health surveillance activities will more accurately define the incidence and risk factors for illness in Oregon, allow identification and treatment of tapeworm carriers, and provide epidemiologic and clinical data to physicians caring for patients in at-risk populations.

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Imported Cutaneous Diphtheria, United Kingdom

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Cutaneous diphtheria is endemic in tropical countries but unusual in the United Kingdom. Four cases occurred in the United Kingdom within 2 months in 2002. Because cutaneous diphtheria causes outbreaks of both cutaneous and pharyngeal forms, early diagnosis is essential for implementing control measures; high diphtheria vaccination coverage must also be maintained.

We report four cases of cutaneous diphtheria that occurred in the United Kingdom during a 2-month period in 2002 and review past cases.

The Study

Patient 1

In September 2002, a 6-year-old girl appeared for treatment with an infected mosquito bite on the ankle and chest lesions. The lesions developed while she was traveling with her mother and four siblings in Bangladesh. Her 12-year-old sister also had infected mosquito bites on her feet. Swab specimens, taken from the chest lesions and feet, were microbiologically cultured. *Staphylococcus aureus, Streptococcus pyogenes,* and toxigenic *Corynebacterium diphtheriae* var *mitis* were isolated from the specimens. The 6-year-old had received flucloxacillin for 5 days before the antimicrobial agent was changed to erythromycin. She had received three primary doses of diphtheria vaccine at birth, 5 months, and 9 months, and a booster vaccination in 2001. The 12- year-old was initially treated as a contact with 7 days of erythromycin; this treatment was extended to 14 days when the laboratory confirmed *C. diphtheriae* infection. She had received three primary diphtheria vaccine doses in 1994, but no booster. The children's mother had received one dose of tetanus-diphtheria (Td) toxoid in July 2002, and their father, a household contact, had no immunization record.The remaining siblings had received at least three primary doses of diphtheria vaccine, and one had received a booster. Nose and throat swabs from all six members of the family, including the two patients, were negative for *C. diphtheriae*. The four contacts were given antimicrobial prophylaxis and completed immunization as appropriate. The two girls were kept home from school until their antibiotic regimen was completed and clearance swabs of the lesions taken 24 hours apart were confirmed as negative.

Ribotyping, a universal molecular typing method for bacteria based upon rRNA gene restriction pattern determination, was performed on the isolates from the two siblings. The ribotype patterns produced were indistinguishable from each other. The girls' schools were asked for lists of all children who had been in contact with the two patients to establish their diphtheria immunization status as shown on the Child Health System (a population-based register of all children living in each locality, which includes information on vaccination status and other health indices). In addition, an information letter was sent to all parents.

Patient 2

In September 2002, an 8-year-old Somalian girl, who had been in the United Kingdom for 4 months, was hospitalized with lesions on her legs and scalp, a sore throat, but no fever. She had no history of diphtheria immunization. The swabs from the throat and lesions yielded toxigenic *C. diphtheriae* var *mitis.* The organism isolated from the throat was only identified because the microbiology department screened all throat swabs routinely for corynebacteria. *S. aureus* was also isolated from the skin lesions. She was treated with flucloxacillin and penicillin. The skin lesions had been swabbed before September but had not been examined for *C. diphtheriae*. The isolates from the throat swab and the lesion were genotyped, and the ribotype patterns that resulted were identical. Two adults and five unvaccinated siblings were identified as contacts. After screening, all were negative for *C. diphtheriae* and were offered vaccination.

Patient 3

At the end of October 2002, an 81-year-old man returned to England from Pakistan with an infected mosquito bite. Toxigenic *C. diphtheriae* var *mitis* was isolated from the lesion. The patient received diphtheria antitoxin and was treated with erythromycin and clarithromycin for 14 days. He had no history of diphtheria immunization. He had traveled alone to Pakistan. Twelve of his close contacts in the United Kingdom required microbiologic screening,

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Table. Distribution by year of cutaneous diphtheria cases caused by toxigenic *Corynebacterium diphtheriae* strains reported in the United Kingdom, 1985–2002

Y	Month	Sex	Age	Biotype	Specimen	Immunization status	Associated country
1985	Sept	M	5	*gravis*	Scalp	4 primary doses	Bangladesh
1990	Oct	F	19	*mitis*	Skin	Unvaccinated	Pakistan
1992	May	F	25	*mitis*	Foot. Insect bites	Unknown	Ghana
1992	July	F	8 mo	*mitis*	Skin	Unvaccinated	Bangladesh
1993	June	F	43	*mitis*	Mosquito bites	Unvaccinated	Gambia
1993	NA[a]	M	20	*gravis*	Skin (RTA)[b]	Unvaccinated	Pakistan
1994	NA[a]	F	64	*mitis*	Foot (leprosy)	Unvaccinated	Bangladesh
1994	Sept	F	32	*mitis*	Foot, throat (membrane)	4 primary doses	India
1995	March	M	27	*mitis*	Skin	4 primary doses	Thailand
1996	Nov	M	27	*intermedius*	Insect bites	4 primary doses	Nepal and Thailand
1997	Dec/Jan	M	39	*mitis*	Foot	4 primary doses + booster	Indonesia
1998	July	M	19	*mitis*	Leg wound	4 primary doses + booster	Tanzania
2000	Nov	F	44	*mitis*	Toe (diabetes)	Unknown	Gambia
2002	Sept	F	6	*mitis*	Chest	3 primary doses	Bangladesh
2002	Sept	F	12	*mitis*	Feet	3 primary doses	Bangladesh
2002	Oct.	M	81	*mitis*	Skin	Unvaccinated	Pakistan
2002	Sept.	F	8	*mitis*	Leg, scalp, throat	Unvaccinated	Somalia

[a]Not available.
[b]Road traffic accident.

although *C. diphtheriae* was not isolated from any. The contacts received erythromycin prophylaxis and were offered vaccine, except for two children who had already been vaccinated. Information also was sent to the patient's contacts in Pakistan.

Conclusions

In the United Kingdom, 17 patients with cases of cutaneous diphtheria due to toxigenic *C. diphtheriae* were reported from 1995 to 2002. All cases were travel-related (Table). Of 15 patients with a vaccination history, 6 were fully immunized (four primary doses by 5 years of age), 2 had received three doses of vaccine, and 7 had not been vaccinated.

In 1985, one patient with a secondary laryngeal case and a total of 16 carriers, including 8 who were secondary contacts of carriers, were associated with one cutaneous case. Dissemination to children and adults in several classes, schools, and households occurred within just 20 days (1). A patient in 1998 generated two asymptomatic carriers. The two sisters in 2002 acquired their lesions at approximately the same time, and thus we cannot determine whether they were infected by the same source or whether one infected the other.

High vaccination coverage is critical. The greater spread of infection after the 1985 case might be related to lower vaccination coverage at that time. In 1985, primary immunization for diphtheria was 85% compared to the current rate of 94%.

Cutaneous diphtheria, still endemic in tropical countries, is the most common nonrespiratory clinical manifestation of infection due to toxigenic isolates of *C. diphtheriae* (2). The disease is characterized by shallow skin ulcers, which can occur anywhere on the body and are usually chronic. They are often associated with infected insect bites, frequently coinfected with pathogens such as *S. aureus* and *S. pyogenes*. Systemic toxic manifestations are uncommon among immunized persons. Skin lesions absorb toxin slowly and can induce high levels of antibodies that produce natural immunization. These lesions are an important reservoir of infection and can cause respiratory and cutaneous infections in contacts as well as outbreaks (3). In several outbreaks, secondary transmission has been higher in contacts of patients with cutaneous infection than in those with respiratory tract infection. Cutaneous diphtheria may also cause greater environmental contamination, through dust and fomites (4).

Cutaneous diphtheria is still being reported in the United Kingdom, even in vaccinated patients and despite high diphtheria vaccination coverage. All cases so far have been acquired in countries where diphtheria is endemic. With increasing travel to and from these countries, more cases may occur. The potential for secondary transmission leads to a large number of contacts requiring follow-up, especially children at school. Moreover, cutaneous diphtheria is likely to be diagnosed less quickly than respiratory infection because the clinical appearance is nonspecific, and other pathogens often coinfect the lesions. Thus, we need to increase the awareness of clinicians and microbiologists of the importance of obtaining swab specimens from any chronic nonhealing skin lesions in patients who have traveled to a disease-endemic area. Wound swab samples from these patients should be examined for *C. diphtheriae*. Early diagnoses and reporting are crucial to trigger effective public health control measures (5).

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Antibiotic Selection Pressure and Resistance in *Streptococcus pneumoniae* and *Streptococcus pyogenes*

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We correlated outpatient antibiotic use with prevalence of penicillin-nonsusceptible *Streptococcus pneumoniae* (PNSP), macrolide-resistant *S. pneumoniae* (MRSP), and macrolide-resistant *S. pyogenes* (MRGAS) in 20 countries. Total antibiotic use was correlated with PNSP ($r = 0.75$; $p < 0.001$), as was macrolide use with MRSP ($r = 0.88$; $p < 0.001$) and MRGAS ($r = 0.71$; $p = 0.004$). Streptococcal resistance is directly associated with antibiotic selection pressure on a national level.

A global trend of increasing antimicrobial resistance, but with wide variations at national levels, is well-documented in the literature (1). Strong evidence supports an association between antibiotic use and resistance in hospitals (2,3). By contrast, the relationship between antibiotic consumption and resistance has been more difficult to establish for the outpatient setting, although some data suggest a direct correlation for streptococcal infections (4–6). For instance, an ecologic study linked penicillin-nonsusceptibility in *Streptococcus pneumoniae* with β-lactam and macrolide use in 12 European countries (7). Recently, McCormick et al. have shown that variation in pneumococcal resistance in the United States is best explained by geographic variation in antibiotic selection pressure, rather than by clonal dynamics (8).

Penicillin-nonsusceptible *S. pneumoniae* (PNSP) and macrolide-resistant *S. pneumoniae* (MRSP) are markers of resistance to antibiotics commonly used as first-line drugs for respiratory tract infections. Although penicillin-resistant *S. pyogenes* has never been observed to date, the increasing rates of macrolide-resistant group A streptococci (MRGAS) pose considerable clinical problems in many countries (4). The aim of this ecologic study was to correlate outpatient antibiotic consumption with reported rates of PNSP, MRSP, and MRGAS in 20 countries on three continents.

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The Study

An ecologic study design was used, which allows measurement of the total (individual and group-level) effect of antibiotic exposure on antimicrobial resistance in streptococci (9). PNSP, MRSP, and MRGAS were chosen as indicator organisms for those effects. National outpatient antibiotic sales data were obtained from published reports, provided by IMS Health Global Services, the National Corporation of Swedish Pharmacies, the Danish Medicines Agency, and the Institute of Public Health in Slovenia (7,10–13). Data for the United States were extracted from IMS data (10). Sales data for total antibiotic use (anatomic therapeutic classification [ATC] group J01) and macrolide use (ATC group J01F) were used to express outpatient consumption in defined daily doses (DDD) per 1,000 inhabitants per day, as recommended by the World Health Organization (WHO) (14).

By using MEDLINE, we performed a systematic search of national and international surveillance studies published in English, French, or German that reported proportional frequencies of PNSP, MRSP, and MRGAS for 1994 to 2000. Representative resistance data from those countries were included for which antibiotic sales data were also available (6,7,10,13,15–34). A time lag of 0 to 2 years between antibiotic sales and ensuing antimicrobial resistance data was considered acceptable for this study. For three countries (Iceland, Finland, and Sweden), the lag time between antibiotic sales data and resistance rates was 0. Studies limited to small geographic areas not representative of a given country and studies limited to particular patient populations were excluded. Nonsusceptibility to penicillin and macrolide resistance included intermediate and high-level resistance.

The relationship between total outpatient antibiotic consumption and rates of PNSP was analyzed for 20 countries (Australia, Austria, Belgium, Canada, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, United Kingdom, and United States). We also calculated relationships between macrolide use and MRSP for 16 countries (Australia, Austria, Belgium, Denmark, Finland, France, Germany, Greece, Ireland, Italy, Netherlands, Portugal, Slovenia, Spain, Sweden, and United Kingdom), and between macrolide use and MRGAS for 14 countries (Australia, Austria, Belgium, Finland, France, Germany, Greece, Italy, Netherlands, Portugal, Slovenia, Spain, Sweden, and United Kingdom). For these correlation analyses, we used two-tailed Spearman coefficients (r) for nonparametric correlations.

Total use of outpatient antibiotics varied from 9.0 DDD/1,000 inhabitants per day in the Netherlands to 36.5 DDD/1,000 inhabitants/day in France (Figure 1). Macrolide use varied from 1.0 DDD/1,000 inhabitants/day in Sweden to 6.0 DDD/1,000 inhabitants/day in France (Figure 2A). Average prevalence of PNSP also showed marked differences, as shown in Figure 1. It was lowest in Scandinavian countries (1% in Norway, 2% in Denmark, 4% in Sweden) and the Netherlands (1%); of medium level in Germany (7%), the United Kingdom (11%), Austria (12%), Belgium, and Italy (both 13%); and high in Portugal (29%), Greece (31%), United States (34%), France (43%), and Spain (50%). Figure 2A illustrates the prevalence of MRSP, which was low in Scandinavia and the Netherlands (3%–5%), medium level in Germany and Portugal (both 9%), and high in Belgium (43%), Spain (36%), and France (53%). MRGAS were distributed in a similar pattern (Figure 2B), with northern European countries showing low levels (0%–4%) and Greece, Italy, and Spain demonstrating the highest levels (29%–38%). Total antibiotic use and prevalence of PNSP were significantly correlated (r = 0.75; $p < 0.001$; Figure 1), as were macrolide use and prevalence of MRSP (r = 0.88; $p < 0.001$; Figure 2A) and macrolide use and prevalence of MRGAS (r = 0.71; $p = 0.004$; Figure 2B).

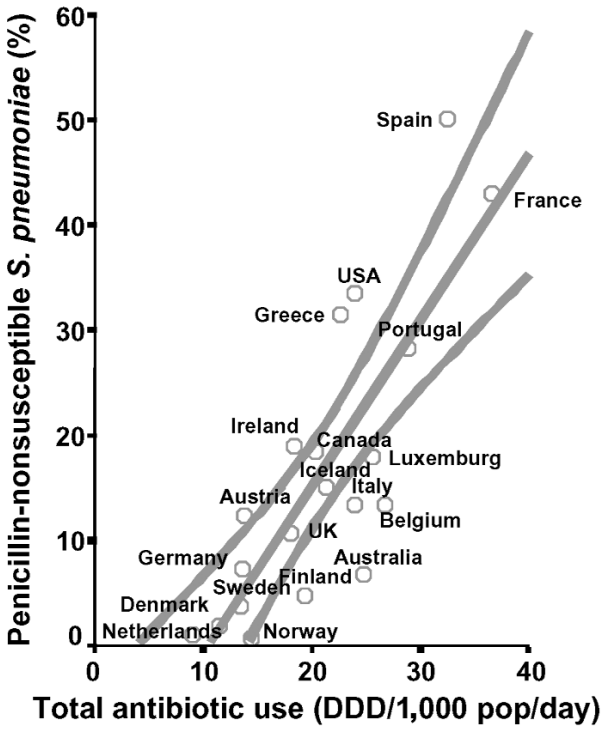

Figure 1. Total antibiotic use in the outpatient setting (vertical axis) versus prevalence of penicillin-nonsusceptible *Streptococcus pneumoniae* (horizontal axis) in 20 industrialized countries. A regression line was fitted with 95% confidence bands (r = 0.75; $p < 0.001$).

Conclusions

This report correlates national outpatient data about antibiotic use with prevalence of antibiotic-resistant *S. pneumoniae* and *S. pyogenes* in Europe, North America, and Australia. The ecologic study design chosen allowed us to evaluate the effect of antibiotic consumption on resistance rates on a national level. A strong relationship was documented between total volume of antibiotic consumption and prevalence of PNSP. An almost linear association existed between macrolide use and proportion of MRSP, a biologically plausible finding that has previously been documented on a smaller scale (6). A weaker, but still significant correlation was found for MRGAS.

We found large differences in resistance rates, even in neighboring countries. A variety of factors are responsible for this, but the selective pressure exerted by inappropriately used antibiotics is likely the most important (35). Indeed, volume of antibiotic use varies widely between countries (12). In previous cross-country comparisons of Germany, France, and the United States (5,30), we suggested that socioeconomic, cultural, and behavioral determinants have a major impact on outpatient antibiotic prescribing practices and resistance prevalence in respiratory pathogens on a national level. In addition, healthcare policies play an important role. In Denmark, for instance, an excessive use of tetracycline was noted, mainly for respiratory tract infections. This situation was corrected by removing subsidization for tetracycline, which resulted in a decrease in its use; this decrease was followed by a parallel decrease in tetracycline resistance among *S. pneumoniae* and other streptococci (36).

Antibiotic use exerts selective pressure on resistance in respiratory pathogens in several ways (35). Any type of recent antibiotic treatment (not only β-lactam agents) can select PNSP by inhibiting susceptible commensal flora and eradicating penicillin-susceptible pneumococci, thereby indirectly promoting the transmission of PNSP and increasing the prevalence of PNSP in a community or country (9). However, whether decreasing antibiotic use in the community will have a sustained impact on resistance rates is unclear. Antibiotic resistance may take longer to return to previous levels than the time it took for antibiotic resistance to increase after excessive antibiotic use. In Finland in the 1990s antibiotic resistance decreased slowly with substantially reduced antibiotic consumption after an initial rapid increase; such situations may lead to different correlations between use and resistance, depending on whether resistance is rising or falling (32).

This ecologic study has some limitations. First, antibiotic sales data cannot be used synonymously with antibiotic exposure without highlighting the problem of patient

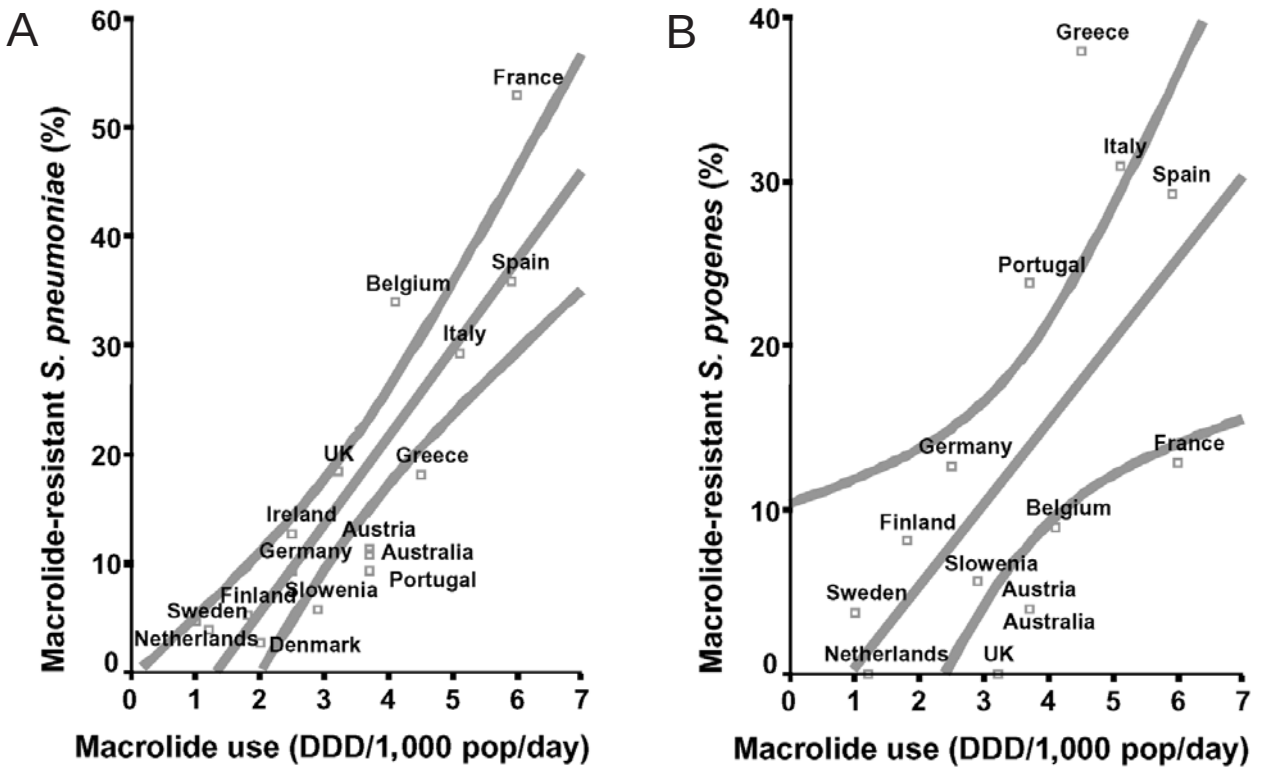

Figure 2. A. Relationship between macrolide use in the outpatient setting (horizontal axis) and prevalence of macrolide-resistant *Streptococcus pneumoniae* (vertical axis) in 16 industrialized countries. A regression line was fitted with 95% confidence bands ($r = 0.88$; $p < 0.001$). B. Relationship between macrolide use in the outpatient setting (horizontal axis) and prevalence of macrolide-resistant *S. pyogenes* (vertical axis) in 14 industrialized countries. A regression line was fitted with 95% confidence bands ($r = 0.71$; $p = 0.004$).

compliance, which is very difficult to quantify. However, in case of low compliance, antibiotics may be used later, and this self-medication may also contribute to resistance. Second, large variations in resistance are typically found, depending on the examined age group, with highest resistance rates and largest amounts of antibiotic consumption usually in children. Due to the study design chosen, we could not account for these differences. Third, no single surveillance study was identified that provided sufficient data on national resistance rates for all countries. Therefore, considerable heterogeneity was found among the quality and characteristics of the included surveillance studies. We tried to minimize this bias by excluding publications not meeting certain standards or examining only selected patient populations. Fourth, the type and number of tested organisms varied widely. Thus, sampling bias may have influenced the results of this study. Finally, data are lacking regarding the maximum time lag between antibiotic consumption in the community and possible changes in resistance patterns on a national level. A period of 1 to 2 years seems plausible, although more rapid changes can be observed.

Further studies should be undertaken to extend our analyses to Asian countries that are demonstrating high rates of streptococcal resistance (31). Moreover, monitoring of antimicrobial resistance should be continued on both the regional and national level. An important role may be played by networks such as the European Antimicrobial Resistance Surveillance System. At the same time, disclosure of sales data through pharmaceutical records may help facilitate research in this area. In summary, the data presented in this ecologic analysis suggest an important association between antibiotic consumption and resistance in streptococcal infections and lend support to the validity of efforts by professional organizations and policymakers to discourage overuse of antibiotics in the community.

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Enterotoxin-producing *Escherichia coli* O169:H41, United States

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From 1996 to 2003, 16 outbreaks of enterotoxigenic *Escherichia coli* (ETEC) infections in the United States and on cruise ships were confirmed. *E. coli* serotype O169:H41 was identified in 10 outbreaks and was the only serotype in 6. This serotype was identified in 1 of 21 confirmed ETEC outbreaks before 1996.

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhea in the developing world and among travelers and is increasingly recognized as a cause of outbreaks in the United States (1). Dalton et al. reviewed confirmed ETEC outbreaks that occurred both in the United States and among passengers on cruise ships that docked in U.S. ports from 1975 through 1995 (2). Twenty-one such outbreaks caused by 17 different ETEC serotypes occurred during this period; 7 (33%) occurred among cruise ship passengers (2). Because laboratory tests for the identification of ETEC are not widely available, outbreaks caused by ETEC may escape recognition, and healthcare workers may miss opportunities for treatment and prevention. To improve recognition of ETEC outbreaks, Dalton proposed that specimens from outbreaks of gastroenteritis that meet certain criteria be referred for ETEC testing at a public health reference laboratory. These outbreaks include those for which routine stool cultures have not yielded an etiologic agent and those which are characterized by an incubation period of 24 to 48 hours, a duration of illness ≥60 hours, and a diarrhea-to-vomiting prevalence ratio of ≥2.5 (2).

The Study

For the 8-year period 1996 through 2003, we reviewed all suspected ETEC outbreaks solely or jointly investigated by the Foodborne and Diarrheal Diseases Branch at the Centers for Disease Control and Prevention (CDC). In accordance with Dalton et al., we defined a confirmed ETEC outbreak as one in which ETEC isolates of the same serotype were isolated from ≥3 ill persons and no other viral or bacterial pathogens were identified, or one in which ETEC isolates of the same serotype were isolated from ≥10 ill persons and no more than one other bacterial or viral pathogen was identified in a single stool specimen. Stool specimens collected during these investigations were routinely cultured for *Salmonella*, *Shigella*, *Campylobacter*, *E. coli* O157:H7, *Yersinia*, and *Vibrio* spp. In many instances, these specimens were also tested for noroviruses.

To identify ETEC, patient specimens were plated to MacConkey agar, and individual colonies or sweeps of confluent growth were tested by polymerase chain reaction (PCR) for heat-labile (LT) and heat-stable (ST) enterotoxin genes (3). Using standard methods, we serotyped LT- or ST-positive isolates for O and H antigens. ETEC isolates were tested by the disk-diffusion method for susceptibility to ampicillin, amoxicillin/clavulanic acid, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole (4). A PCR–restriction fragment length polymorphism test was used to identify the H41 gene in selected nonmotile *E. coli* O169 isolates (5).

Conclusions

During the 8-year study period, CDC received isolates from 59 outbreaks for ETEC testing. Sixteen met the criteria for our definition of a confirmed ETEC outbreak; three occurred on international cruise ships that docked in U.S. ports and 12 occurred in the United States (Table). We identified ETEC in specimens from six other outbreaks that did not meet these criteria, either because ETEC isolates of the same serotype were isolated from only two persons (n = 2) or because we isolated additional bacterial pathogens from the specimens (n = 4).

The 16 ETEC outbreaks had a median of 41 ill persons per outbreak (range 5–916), for a total of 2,865 patients. From 81% to 100% of ill persons in each outbreak reported diarrhea; less frequently reported symptoms included abdominal cramps (66%–90%), fever (0%–73%), nausea (44%–70%), and vomiting (0%–33%). In the 15 outbreaks in which diarrhea and vomiting were reported, the median "diarrhea-to-vomiting prevalence ratio" (the percentage of patients who reported diarrhea divided by the percentage of patients who reported vomiting) was 7.7 (range 2.9–undefined: the upper limit of the range is undefined because in two outbreaks all ill persons interviewed denied vomiting). In nine outbreaks with sufficient data, incubation periods among individual patients were 5–158 hours. The median incubation period was 24–48 hours for 10 of the 12 outbreaks for which it could be calculated. The

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Table. Characteristics of enterotoxigenic *Escherichia coli* (ETEC) outbreaks,[a] United States, 1996–2003

No.	Mo/y	Location	Setting (reference)	Presumed source	No. ill	% Diarrhea (bloody)	% Vomiting	Median incubation, h (range)	Median illness duration, days (range)	No isolates	Serotype/toxin type[b]	Antimicrobial resistance[c]
1	3/96	Caribbean	Cruise ship	Drinking water	652	98 (9)	19	–	–	6	O169:H41/ST	Tc
										1	O6:H16/ LT,ST	Sensitive
										1	O27:H7/ST	St, Su, Tc
										1	O34:H10/ST	Tc
2	4/97	Caribbean	Cruise ship (5)	Drinking water, ice	429	100 (6)	14	–	>3	8	O169:H41/ST	Tc
										3	O148:H28/ LT,ST	Tc
										1	O78:H12/ST	Ch, St, Su, Tc, TmS
										1	O27:H7/ST	St, Su, Tc
3	4/97	California	Restaurant	Beans, enchilada, tacos, rice, tortilla chips	41	95	17	24 (5–85)	2 (0.3–4)	11	O27:H7/ST	Sensitive
4	6/97	Massachusetts	Boxed lunch	Tomato, mozzarella salad	33	97	33	48 (24–96)	3.7 (1–7)	5	O25:NM/ST	Sensitive
5	7/97	Minnesota	Catered party	Fresh vegetables	15	100	13	37 (8-158)	3.2 (0.4–6)	4	O169:H41/ST	Tc
6	4/98	Mexico-Hawaii	Cruise ship	–	397	96	33	–	–	3	O6:H16/ LT,ST	Sensitive
										1	O6:H16/ LT,ST	St, Tc
										2	O169:H41/ST	Tc
										1	O148:H28/ LT,ST	St, Su, Tc
										1	O148:H28/ LT,ST	Tc
										1	O27:H7/ST	St, Su, Tc
7	6/98	Illinois	Catered parties	Potato, macaroni, egg salads	916	100	8	50 (40–76)	5 (2–9)	11	O6:H16/ LT,ST	Sensitive
8	8/98	Minnesota	Restaurant (6)	Parsley	66	100 (0)	6	25	8	7	O6:H16/ LT,ST	Ap, St, Su, Tc
										1	O159:H4/LT	Ap, St, Su, Tc, TmS
										1	O27:H7/ST	St, Su, Tc
9	9/98	Minnesota	Restaurant	–	5	100 (0)	0	48	6	3	O169:H41/ST	St, Su, Tc
10	5/00	Washington	Cruise ship	Basil	100	100	5	40 (27–67)	10 (0.5–21)	3	O169:H41/ST	Tc
11	6/00	New York	Banquet	–	40	97 (0)	3	48 (24–96)	3 (1–8)	5	O169:H41/ST	Tc
12	7/00	Utah	Wedding rehearsal	–	45	100	29	33 (18–59)	2.3 (1–3)	5	O27:H7/ST	St, Su, Tc
13	7/01	Wisconsin	Catered party	Quesadillas, fajitas, nacho chips, beans	21	100	–	38 (9–70)	6 (1–8)	3	O169:H41/ST	Tc
14	8/01	Illinois	Catered party	–	24	100	0	–	–	3	O169:H41/ST	Ap, Tc
										1	O169:H41/ST	Tc
										2	O6:H16/LT,ST	Sensitive
										1	O25:NM/ST	Ap
15	10/02	Oregon	Catered party	Garlic chicken lasagna	40	98 (13)	15	72 (24–144)	5	3	O27:H7/ST	St, Su,Tc
16	8/03	Tennessee	Catered party	Catfish, coleslaw	41	81 (0)	5	2	2.5	12	0169:H49	Tc

[a]An outbreak is defined as ≥3 ill persons infected with the same ETEC serotype and no other viral or bacterial pathogens, or ≥10 ill persons with the same serotype and no more than one other bacterial or viral pathogen identified.
[b]NM, nonmotile; LT, heat-labile toxin; ST, heat-stable toxin.
[c]Ap, ampicillin; Ch, chloramphenicol; St, streptomycin; Su, sulfisoxazole; Tc, tetracycline; TmS, trimethoprim-sulfamethoxazole.

duration of illness, reported in 11 outbreaks, was 0.3–21 days. The median duration of illness was ≥60 hours in 11 of the 13 outbreaks for which it could be calculated.

A vehicle was implicated in 11 (69%) outbreaks. Unbottled ship's water or beverages containing ice prepared on board the ship were implicated in 2 of the 3 outbreaks on cruise ships that had docked in foreign ports. The source of the third international cruise ship outbreak was not determined. Although no problems with chlorination of bunkered water were documented in the two outbreaks, this problem has been seen in previous waterborne ETEC outbreaks aboard cruise ships (6). Basil served on board ship was implicated as the source of ETEC in the remaining cruise ship outbreak (on a ship that docked only

in U.S. ports). One other outbreak was attributed to a fresh herb (parsley) served raw (7). Salads made with raw vegetables were implicated in four other domestic outbreaks.

If we define a strain as each ETEC serotype identified during an outbreak that has a unique antimicrobial resistance pattern, we identified a total of 30 strains representing eight different serotypes in specimens from the 16 outbreaks (Table). In five outbreaks, we isolated more than one ETEC serotype. Heat-stable toxin (ST)-producing *E. coli* O169:H41 was the most commonly identified serotype. This serotype was identified as the only pathogen in specimens from six outbreaks in the United States and was identified along with other ETEC serotypes in four additional outbreaks. Three of these four outbreaks occurred on international cruise ships. In 21 previously reported ETEC outbreaks, *E. coli* O169:H41 had been isolated only once, from an outbreak that occurred among international cruise ship passengers in 1995, in which another ETEC serotype predominated (2). By all of the basic epidemiologic and clinical characteristics that we analyzed, outbreaks in which *E. coli* O169:H41 was identified alone, or in combination with other serotypes, did not appear to differ from outbreaks in which this emerging strain was not identified.

Resistance to antimicrobial agents remained common among ETEC isolates (Table). Twenty-four (80%) of the 30 strains were resistant to tetracycline, 11 (38%) were resistant to sulfisoxazole, 4 (13%) were resistant to ampicillin, and 2 (7%) were resistant to trimethoprim-sulfamethoxazole. All strains of O169:H41 were resistant to tetracycline, and two were also resistant to at least one additional antimicrobial drug. Only two outbreaks were caused exclusively by pan-sensitive ETEC strains (7%).

A comparison of ETEC outbreaks reported to CDC from 1996 through 2003 with those from previous years shows that outbreaks on cruise ships and in the United States continue to occur and that antimicrobial resistance among ETEC isolates remains common. Raw vegetables and herbs have been increasingly implicated as the vehicles for ETEC outbreaks in recent years. This finding is in keeping with an increase in produce-associated outbreaks among other foodborne bacterial pathogens (8). Finally, a new ETEC serotype, ST-producing O169:H41, has become predominant.

The first report of O169:H41 was in association with a foodborne outbreak in 1991 in Japan, where this serotype continues to be isolated (9–11). Hamada reported four outbreaks that occurred from June 1997 to August 1998; the largest of these outbreaks had a 57% attack rate and resulted in approximately 2,800 cases. All four outbreaks occurred at either restaurants or catered events (11). Contaminated wakame seaweed was implicated in one of the outbreaks and considered the likely cause in another (11). Nishakawa et al. report that O169:H41 has become the most prevalent ETEC serotype in Japan (12).

In 1995, CDC detected this serotype for the first time during an outbreak on a Caribbean cruise ship. It was identified during two additional Caribbean cruise ship outbreaks before causing a domestic outbreak in Minnesota in 1997. In addition to being identified in 10 of the 16 ETEC outbreaks that met our criteria, O169:H41 was also a predominant serotype in 4 of the 6 ETEC outbreaks that did not meet our criteria for a confirmed outbreak, either because the serotype was isolated from two persons only or because an additional pathogen was also isolated in the outbreak.

The emergence and eventual predominance of O169:H41 in the United States and Japan may have important implications for ETEC vaccine producers. Nishikawa et al. characterized strains of O169:H41 from Japan and reported that they are not clonal and that they possess a novel colony-forming factor (12).

From 1996 through 1999, laboratory-confirmed ETEC outbreaks represented 0.2% of all foodborne outbreaks reported to CDC (13). This number is likely to be an underestimate because special diagnostic tests are required to confirm ETEC. Seventy-one percent of outbreaks of foodborne illness reported to CDC during this period were of unknown cause. In a previous study, Hall et al. demonstrated that the epidemiologic and clinical syndrome in 1.1% of outbreaks of unknown cause reported to CDC from 1982 through 1989 was compatible with infections caused by ETEC or STEC (14). ETEC is also responsible for some episodes of sporadic diarrheal disease. When researchers systematically looked for it, they isolated ETEC from 1.4% of stool samples from patients visiting urban and rural health maintenance organization clinics in Minnesota for diarrhea (15). Our data suggest that the clinical criteria proposed by Dalton et al. for suspecting ETEC as a cause of an outbreak of unknown cause (median incubation 24–48 hours, mean or median duration >60 hours, and diarrhea-to-vomiting prevalence ratio $\geq$2.5) remain valid.

The epidemiology of ETEC outbreaks in the United States is changing, but the incidence of these outbreaks does not appear to be decreasing. Researchers cannot use routine stool cultures to detect ETEC, and delays in stool sample collection for $\geq$7 days greatly reduces yield (16,17). For outbreaks that meet the clinical profile and for which routine stool diagnostic tests have not yielded an enteric pathogen, physicians and public health authorities should send *E. coli* isolates to reference laboratories, such as CDC, for ETEC testing.

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Adherence Barriers to Antimicrobial Treatment Guidelines in Teaching Hospital, the Netherlands

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To optimize appropriate antimicrobial use in a university hospital and identify barriers hampering implementation strategies, physicians were interviewed regarding their opinions on antimicrobial policies. Results indicated that effective strategies should include regular updates of guidelines that incorporate the views of relevant departments and focus on addressing senior staff and residents because residents do not make independent decisions in a teaching-hospital setting.

In an era of increasing bacterial resistance and the availability of a plethora of antimicrobial agents, hospitals have developed policies to promote prudent antimicrobial prescribing (1). The mainstay of such policies is preferably an evidence-based antimicrobial treatment guideline (2). Adherence to such hospital guidelines is often low to moderate (40%–60%) (3,4). Therefore, much effort is put into programs aimed at optimizing the antimicrobial prescribing practices of physicians. To plan an effective intervention strategy, however, one must know the extent to which clinicians perceive the need for a guideline and support implementing that specific guideline (5). The impact of different implementation strategies varies and when, and under what conditions, a particular strategy should be used is often not clear (1,3,4).

We examined barriers that existed in different groups of physicians to the use of a general, hospitalwide antimicrobial treatment guideline. A qualitative approach was chosen to maximize the identification of relevant issues, especially on content and development process of the guideline and physicians' and organizational characteristics (6,7).

The Study

Physicians were asked on their opinions on antimicrobial policies in general and on aspects of the current antimicrobial treatment guideline and its usefulness in daily clinical practice, by using in-depth interviews lasting 20–45 minutes. That antimicrobial treatment guideline was drawn up by the hospitals' antibiotic use committee, which was composed of specialists of relevant departments. Paper copies of the antimicrobial treatment guideline were distributed hospitalwide, in 1995, with an update in 1999. From the Department of Internal Medicine of the University Hospital, Groningen, physicians were recruited through their chief medical officers in October and November 2001. Interviewees were not paid; all involved were informed that interview data would be strictly confidential to guarantee interviewees independence. One resident and one supervisor were interviewed from each of six internal medicine subspecialties—intensive care, general internal medicine, pulmonology, gastroenterology, nephrology, and hematology. Residents had 1–6 years of precertification training, and supervisors had been board-certified for 1 to 23 years as a specialist. From the group of infectious disease consultants, two clinical microbiologists and a consulting infectious disease specialist were interviewed. Each interview was concluded with a case-scenario to explore agreement between general opinions on antimicrobial use and response to a specific infectious disease case.

Interviews were audiotaped and transcribed verbatim; the content was analyzed by P.M. and W.R. One recording of an interview with a clinical microbiologist was damaged and could not be used. Recurrent topics were attributed to dominant themes. Important issues and themes emerging from previous interviews were incorporated into subsequent interviews. Themes were classified as barriers related to 1) the guideline, 2) physicians' characteristics, and 3) characteristics of the institution. Interviewing and analysis were partly simultaneous, which is consistent with the grounded theory approach (8). Physicians were interviewed once. After 15 physicians had been interviewed, no new issues came up, and we stopped interviewing.

Barriers Related to the Guideline

All physicians but one were aware of the guideline, although six never had received a personal copy (Table 1). They suggested that more effort should be put into familiarizing physicians with the guideline. Residents preferred an electronically available copy of the guideline. All physicians agreed with the basic principle of the guideline: an initially empirical antimicrobial treatment should be streamlined to the most narrow-spectrum antimicrobial agent effective against isolated pathogens. Physicians stressed that the guideline needed to be consistent with existing policies, concise, and up-to-date. Supervisors' expected their own prescribing to be consistent with the guideline, without actually knowing its contents, though

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Table 1. Quotations illustrating themes

Level of	Quotation
Guideline characteristics	"It would be great to have an electronic version" or "…even better everyone | would have| a handheld PDA with guideline." [R1][a]
	"You have to go with the flow and not necessarily against it…otherwise you will have a hard time |getting the guideline accepted|.[b]" [S1]
Physicians' characteristics	"I often look in the booklet |guideline| especially for those indications that you do not encounter much…" [R4]
	"…you sometimes get these incomplete results: just a gram-stain without sensitivity results, I then rather wait for the complete results" [R5]
	"…they |residents| do not look at the quality of the |culture and sensitivity| tests" [ID]
	"I would continue. |with broad-spectrum therapy even when a cultured pathogen is sensitive to a more narrow spectrum agent| The patient is doing well, he is responding to his antibiotic therapy. I would not streamline at this time. I do not see any reason. ...Never change a winning team." [R1]
	"My autonomy… as a lung specialist I feel I have and can decide on |what treatment for| pulmonary infections" [S2]
	"…for the large majority of patients it |choice of antibiotic| is just very clear, it is just a formality." [S3]
	"In our department? I cannot remember the time we had resistant pneumococci. We know they exist and we remind each other of that, but we have never had that here." [S2]
	horrible...I can imagine obliging people to use it |registration form| in the framework of their training… but to have to defend this as a standard measure, I do think this goes too far." [S5]
	"A wonderful idea… it could be quite a guiding instrument ... it should be education and intervention." [ID]
Social and institutional context	"…and when you move to another department you learn within a week their |supervising specialists| prescribing preferences… they will just keep an eye on you." [R4]
	"I would first consult my supervisor and if he gets stuck I would ring up a microbiologist." [R5]

[a]R, resident; S, supervisor; ID, infectious disease specialist.
[b]\|Text\| is added by the author for clarification. This additional text is merely meant to clarify a physician's statement, and we have made every effort to not alter the implication of any statement.

residents experienced the opposite: residents experienced that supervisors regularly prescribed or advised them to prescribe antibiotics that were not recommended by the hospital guideline. Infectious disease consultants, as members of the antibiotic use committee, had contradictory views on one aspect of the contents of the guideline. They supported its recommendations for using aminoglycosides when appropriate but were reluctant to advise prescribing them for individual patients.

Barriers Related to Physicians' Characteristics

Residents were more receptive to using the guideline than were supervisors, especially for rare infectious diseases because they lack experience and have to look up the most effective therapy for a specific condition more often. Junior residents acknowledged a lack of knowledge in interpreting culture and antimicrobial sensitivity test results, resulting in problems with effectively using the guideline based on such tests (Table 1). Infectious disease consultants shared this concern. In contrast to their statements supporting streamlining antimicrobial therapy, residents reported that they were not inclined to change therapy with an effective broad-spectrum antimicrobial agent, once the pathogens' sensitivity test results became available.

Supervisors did not perceive a strengthened antibiotic policy as an advantage because they considered guidelines a threat to their professional autonomy and as interfering with daily clinical practice. Prescribing an antimicrobial agent was often considered a routine activity. Supervisors doubted the need for an antimicrobial use policy, which was reinforced by the fact that they did not perceive many problems with antimicrobial resistance in daily clinical practice.

At the time of the interviews, a paper critical-pathway[1] was discussed as a possible decision support tool for improved antimicrobial therapy. Supervisors and residents were negative towards such a tool. Supervisors considered it an unnecessary and unacceptable infringement of daily clinical work, while residents were mostly concerned about the added paperwork. The infectious disease consultants had great trust in a critical-pathway to guide antimicrobial drug prescribing, welcoming its educational value and potential for improving actual prescribing behavior.

Social and Institutional Context

Residents in most teaching hospitals are not independent decision makers, and experienced specialists supervise their prescribing choices (Table 1). Residents run the day-to-day clinical care of patients in our hospital; they rotate to different departments at 4-month intervals and have to adapt each time to the mores of a new department or supervisor. They considered the antimicrobial-treatment

[1]A paper "critical-pathway" combines an antimicrobial drug order form with a decision support tool. Filling out a few relevant case-characteristics guides the prescriber to the guideline's recommendation for that specific case.

guideline a helpful tool in coping with existing differences between departments; some departments had their own protocols but mostly discussed antimicrobial use policies informally in departmental patient reviews. The role of the infectious disease consultant was one of adviser. Residents would primarily seek advice from their supervisor, and the final decision is always made by the supervisor.

Case Scenario

To further ascertain the physicians' use of the antibiotic treatment guideline, we presented a scenario for a case of community-acquired pneumonia (Appendix). All physicians, except for one supervisor, began the patient's treatment with broad-spectrum antimicrobial agents. Residents were hesitant to streamline initial therapy, fearing that such changed therapy might be clinically less effective. Infectious disease consultants and supervisors streamlined therapy based on gram-stain results only.

Conclusion

Our findings support earlier study findings that an intensive implementation strategy is needed for physicians to make their prescribing practices consistent with guideline recommendations. Table 2 shows the identified barriers along with our suggestions about which interventions might be effective. Any implementation process passes through different stages, each requiring a different intervention approach (9). The supervisors are in an early stage of such a process; they need to be motivated to use the antimicrobial-treatment guideline and to change their prescribing behavior accordingly. Clear involvement in the development of the antimicrobial-treatment guideline may overcome reservations of supervisors with regard to feelings of losing their autonomy. Supervisors see no need to follow the guideline recommendations; they do not perceive antimicrobial resistance as a problem, which may be understandable in view of the low resistance patterns in Dutch hospitals (10). Their routine decision-making leaves little room for guideline consultations. Providing feedback on their own and departmental prescribing patterns may identify areas to be improved and raise awareness of a need to change (11,12). The usefulness of the guidelines could be emphasized for nonroutine cases, about which physicians were less reluctant to consult the guideline.

Residents are more open to using the guidelines; they are willing to adopt the recommendations because it helps them in their learning process, making them ideal candidates for interventions. For them, the barrier to be addressed is whether streamlining is safe. One way of affirming this is facilitating a better understanding of culture and sensitivity tests, for example, through infectious disease consultants' support (6). As paper critical-pathways will not suffice, face-to-face educational visits, so-called academic detailing, may be a better way to improve residents' prescribing practices (13). Academic detailing should focus not only on interpretation of test results but also on acting on the implications. Infectious disease consultants should be motivated to give advice consistent with the guideline.

In an institutional context where residents are not independent decision makers, any implementation plan should combine strategies aimed at both residents and supervisors. For residents who change departments regularly, a generally adopted hospitalwide guideline facilitates a consistent learning environment and increase their rational decision making. Addressing the role model function of supervisors for residents may be one more way to motivate

Table 2. Barriers and proposed interventions

	Barriers identified	Proposed interventions
Guideline	1. Dissemination	1. Develop and actively distribute hard-copy and electronic version
		2. Incorporate departmental policies, and update regularly
	2. Credibility of content	– For both 1 and 2, organize meetings to introduce guidelines and set up an active outreach committee
Physician	Readiness to change or use the guideline	
	Supervising specialists	
	3. No need for a guideline, because	3. A combination of group and individual feedback ("academic detailing") to supervisors and residents
	– Routine prescribing	
	– No perceived resistance problems	
	4. Autonomy	4. Incorporate specialists/departmental views in guideline (see 2, above)
	Residents	
	5. Insufficient knowledge	5. Active educational support on interpretation of culture-results and for streamlining therapy
	– Of culture results	
	– Low self-efficacy regarding streamlining	
	Infectious disease consultants	
	6. Overestimate the feasibility of an intervention	6. Check support before implementation of an intervention
Social and institutional context	7. Residents are not independent decision makers and their prescribing decisions are supervised by specialists	7. Target both residents and supervising specialists
	8. Infectious disease consultant secondary to supervisor	8. Target supervisor, formalize advice of consultants
	9. Different guidelines between departments	9. Incorporate departmental policies (see 2 and 5, above)

them to use the guideline, in view of the impact that supervisors have on residents (14).

The limited number of physicians interviewed in this study is in line with a qualitative research approach aimed at generating hypotheses (15). We found physicians to be very open in expressing their sometimes negative views during the interview sessions. Residents were quite frank about their relationship with their supervisors, possibly because the interviewer had no direct link to any chief medical officer and confidentiality was assured.

In conclusion, intervention strategies should focus on improving dissemination and credibility of the recommendations, focusing on both supervisors and residents, although each group needs a tailored approach. Active outreach, as in face-to-face educational visits, may be the best approach to tackling the various barriers in one intervention program aimed at optimizing antimicrobial use.

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Appendix: Case Scenario

The following scenario was presented for a case of community-acquired pneumonia.

A male patient aged 63 years, with hypertension treated with metoprolol and hydrochlorothiazide, is referred by a local general practitioner to the emergency department of your hospital at 11:30 p.m. He has a temperature of 40°C and is dyspneic but not confused. Physical examination reveals only crackles and egophony in the right lower lung field.

Question (Q) 1. What additional examinations would you request?

Q2. What kind of therapy would you suggest?

The next morning a chest x-ray shows infiltration in the right lower lung field, and the sputum culture shows gram-positive diplococci.

Q3. Does this influence your therapeutic decision?

Two days later the patient is improving and the fever has subsided. Blood-culture results read literally as follows: *Streptococcus pneumoniae*, sensitive to penicillin G, amoxicillin, amoxicillin with clavulanic acid, and cefuroxime.

Q4. Do you adapt your therapeutic choice?

Hospital Guideline on Community-Acquired Pneumonia

For this straightforward case of a patient with a clearly community-acquired pneumonia, the initial empiric treatment according to the hospital guideline would be amoxicillin/clavulanic acid or cefuroxime. The guideline recommends streamlining antimicrobial therapy to intravenous penicillin G or oral amoxicillin based on sensitivity tests of the isolated *S. pneumoniae.*

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Neisseria meningitidis C:2b:P1.2,5 with Intermediate Resistance to Penicillin, Portugal

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For 1 year, serogroup, serotype, serosubtype, and penicillin susceptibility of meningococci circulating in various regions in Portugal were evaluated. Most frequent phenotypes were B:4:P1.15 (13.4%) and C:2b:P1.2,5 (75.9%), which are also common in Spain. Overall, 27.5% of C:2b:P1.2,5 strains showed intermediate resistance to penicillin. Laboratory-based surveillance of meningococcal infection in Portugal provides important information to assess the adequacy of public health measures.

In Europe, infections caused by *Neisseria meningitidis* are associated with high rates of disease and death (1). Outbreaks of invasive meningococcal disease, including some cases of sudden death in some countries, suggest that vaccination may be necessary to reduce the incidence of this infection (2). Knowledge of the antigenic characteristics of *N. meningitidis* would help determine the most appropriate control strategies, which may vary from country to country.

Serogroups B and C are the most widespread, representing approximately 95% of cases of invasive meningococcal disease in Europe (1), and serogroup B is the most frequent cause of invasive meningococcal disease both in America and Europe (3). Serotypes and serosubtypes can be distinguished according to antigenic variants of the outer membrane proteins PorB and PorA (4).

In Portugal, the Compulsory Notifiable Diseases system is used, and meningococcal disease is a notifiable disease (5). The serogroup C conjugate vaccine was licensed in 2000, and vaccination has been voluntary since the third quarter of 2001. Laboratory analysis of serotypes and serosubtypes, not previously studied in meningococcal isolates from Portugal, would contribute to understanding meningococcal diversity and spread of meningococcal disease before the voluntary vaccination period.

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We report a laboratory-based surveillance study of the *N. meningitidis* serogroups, serotypes, and serosubtypes in circulation in Portugal, as isolated from patients with cases of cultured-confirmed invasive infection. Susceptibility to penicillin was also evaluated.

The Study

A total of 116 isolates of *N. meningitidis* were detected through a laboratory surveillance study; 27 hospitals from different regions of Portugal participated. The investigation was conducted for 12 consecutive months (September 2000 to August 2001) after a 2-month pilot study (July and August 2000) in six hospitals. The catchment population of the participating hospitals included approximately 7,400,000 residents (71% of the Portuguese population).

Isolates of *N. meningitidis* were directly submitted at –20ºC to the Antibiotic Resistance Unit in National Institute of Health Dr. Ricardo Jorge, Lisbon, as pure isolates. However, in some cases, only primary cultures performed at participant hospitals were sent, at 35ºC, and isolated in Antibiotic Resistance Unit. All strains were identified by standard methods (6).

The inclusion criteria for laboratory diagnosis were as follows: nonrepetitive and consecutive blood, cerebrospinal fluid (CSF) samples, or both in persons with symptoms compatible with invasive meningococcal disease. The strains from the pilot study were also included in the analysis. A case of invasive meningococcal disease was defined as disease in which *N. meningitidis* had been

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isolated by culture from two normally sterile sites (blood and CSF) in a resident of the surveillance area.

Serogroup was determined on 116 isolates by slide agglutination with the polyclonal specific rabbit antisera for A, C, X, Y, Z, W135, and 29E capsular polysaccharides of *N. meningitidis* and the monoclonal antibodies to determine serogroup B (Murex Diagnostic, Dartford, U.K.) (6). Serogroup findings by slide agglutination were checked by polymerase chain reaction with previously described primers (7).

For serotype and serosubtype analysis and susceptibility testing, 109 strains were available. Serotypes and subtypes were determined by an enzyme-linked immunosorbent assay method (4) by using monoclonal antibodies (RIVM, National Institute of Public Health and the Environment, the Netherlands). Serotype-specific reagents included 1, 4, 2a, 2b, 14, and 15; serosubtype-specific reagents included P1.1, P1.2, P1.4, P1.5, P1.6, P1.7, P1.9, P1.10, P1.12, P1.13, P1.14, P1.15, and P1.16.

The susceptibility to penicillin (Wyeth Lederle Portugal, Algés, Portugal) was assessed by determining MICs by using the agar dilution method with Mueller-Hinton agar supplemented with 5% sheep blood and incubated in 5% CO_2 at 35°C for 24 h. *N. meningitidis* strains with an MIC of penicillin of ≤0.06 μg/mL were susceptible, strains with an MIC of penicillin of 0.12 μg/mL to 1 μg/mL had intermediate resistance to penicillin. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as control strains. Production of β-lactamase was tested by nitrocefin (a chromogenic cephalosporin method).

The study sample included slightly more serogroup C (58/116, 50.0%) than serogroup B (55/116, 47.4%) isolates; the rest were serogroup W135 (3/116, 2.6%). The most frequent type and subtype of serogroup B was 4:P1.15 (13.4%) and of serogroup C was 2b:P1.2,5 (75.9%) (Table). Phenotype C:2b:P1.2,5 accounted for 37.6% of all strains, but no differences existed by age and region (data not shown). Most of the 41 strains with this phenotype were isolated from November 2000 through June 2001, with a peak from February 2001 to April 2001 (Figure). This peak contributed to the higher number of isolates obtained during this period.

Strains with intermediate resistance to penicillin from serogroup B had the following serotypes and subtypes: 4:P1.15 (two strains) and one strain of each 1:P1.15, 4:P1.2,5, 14:P1.12 and NT:P1.15. Strains with intermediate resistance to penicillin from serogroup C had the following serotypes and subtypes: 2b:P1.2,5 (30 strains) and 2b:P1.2 (2 strains) and 2b:NST (2 strains). One strain from the phenotype W135:2a:P1.2,5 also showed intermediate resistance to penicillin (Table).

Conclusions

This study, covering approximately 71% of the Portuguese population, shows that serogroups B and C are dominant in Portugal, as they are elsewhere in Europe (1). Meningococcal disease in our study followed the usual European pattern (1), including the seasonal peak in winter and the age distribution, with children <5 years of age being the most affected group (data not shown).

B:15:P1.7,16 and B:4:P1.4 were the fourth and fifth most frequent serotype and subtypes of serogroup B in our sample, respectively, but were the major phenotypes of invasive meningococci in other European countries in 1999 and 2000, followed by B:4:P1.15, B:1:P1.14 and B:4:P1:10, in descending order (1,9). However, the most frequent serotype and subtype of serogroup B in this study was 4:P1.15 (13.4%), as in Spain (1). Spain was the only European country to report phenotype B:4:P1.15 from 1993 to 1996 (9), but it was found in the Czech Republic, Slovakia, and Malta in 1999 through 2000 (1).

Between serogroup C strains, those with serotype and subtype 2b:P1.2,5 (75.9%) were the most frequent, followed by 2b:P1.2 (7.5%) (1,9). In Europe, in 1999 through 2000, the prevalent phenotypes for strains of serogroup C were C:2a:P1.5 and C:2a:P1.2,5, followed by C:2b:P1.2,5, C:2b:P1.2, and C:2a:P1.2 (1).

The apparent endemicity of *N. meningitidis* C:2b:P1.2,5 in Portugal (Figure) (8), as in Spain, suggests that the features of the Iberian Peninsula are favorable for this phenotype. Invasive meningococcal disease may be caused by host factors more than bacterial determinants (10,11). Molecular analysis may be able to indicate whether the phenotype 2b:P1.2,5 is a variant of clones spreading in other countries or even a variant of previously identified clones in Portugal. Monitoring the incidence of this phenotype and its apparent emergence from 1995 (33.3%) to 2001 (75.9%) (Table) (8) is important.

The three strains of serogroup W135 in this study were all serotype and subtype 2a:P1.2,5, the predominant clone in Europe (1). This serotype was the cause of an outbreak in 2000, after the Hajj pilgrimage to Mecca (12). We have no information about contact between patients and Hajj pilgrims or the European outbreak. However, the main serotype and subtype in serogroup W135 in Portugal previously (between 1995 and 1999) was also 2a:P1.2,5 (Table).

The emergence of serogroup C, including numerous isolates with intermediate resistance to penicillin (13,14), leads us to emphasize the importance of the prophylaxis and the need for cost-benefit studies to control meningococcal disease. Resistance to penicillin can impede treatment of invasive disease, making surveillance of this resistance important. Strains of phenotypes B:4:P1.15 and C:2b:P1.2,5 most frequently had intermediate resistance to

Table. Distribution of 109 invasive meningococcal isolates by serogroup, serotype, serosubtype and susceptibility to penicillin

Serogroup (no. of strains)	Serotype (no. of strains)	Serotype: subtype[a]	No. of strains (%)	Penicillin susceptibility		No. of strains with the same phenotype in 1995–1999[b,c]
				MIC (µg/mL)	S, IR[b]	
B (n = 52)	1 (n = 12)	1:NST	6 (11.6)	0.06	S	
		1:P1.13	2 (3.8)	0.06	S	
		1:P1.14	1 (1.9)	0.06	S	
		1:P1.15	1 (1.9)	0.25	IR	
		1:P1.4	1 (1.9)	0.06	S	
		1:P1.9	1 (1.9)	0.06	S	
	2b (n = 1)	2b:NST	1 (1.9)	0.06	S	
	4 (n = 16)	4:P1.2,5	2 (3.8)	0.06	S	
			1 (1.9)	0.125	IR	
		4:P1.4	3 (5.8)	0.06	S	
		4:P1.6	1 (1.9)	0.06	S	
		4:P1.14	1 (1.9)	0.06	S	1 (IR)
		4:P1.15	5 (9.6)	0.06	S	1 (S)
			2 (3.8)	0.125	IR	
		4:NST	1 (1.9)	0.06	S	
	14 (n = 2)	14:NST	1 (1.9)	0.06	S	3 (S)
		14:P1.12	1 (1.9)	0.25	IR	
	15 (n = 6)	15:P1.7	1 (1.9)	0.06	S	
		15:P1.7,16	5 (9.6)	0.06	S	5 (S)
	NT (n = 15)	NT:P1.2,5	1 (1.9)	0.06	S	
		NT:P1.6	1 (1.9)	0.06	S	2 (S)
		NT:P1.7,16	1 (1.9)	0.06	S	1 (IR)
		NT:P1.9	5 (9.6)	0.06	S	1 (S)
		NT:P1.12	1 (1.9)	0.06	S	
		NT:P1.15	2 (3.8)	0.06	S	1 (S)
			1 (1.9)	0.25	IR	
		NT:NST	3 (5.8)	0.06	S	1 (IR)
C (n = 54)	2a (n = 2)	2a:P1.2,5	1 (1.9)	0.06	S	2 (S)
		2a:P1.5	1 (1.9)	0.06	S	
	2b (n = 48)	2b:P1.2	2 (3.7)	0.06	S	1 (IR)
			1 (1.9)	0.125	IR	
			1 (1.9)	0.25	IR	
		2b:P1.2,5	11 (20.4)	0.06	S	18 (3 S, 15 IR)
			10 (18.5)	0.125	IR	
			18 (33.3)	0.25	IR	
			2 (3.7)	0.5	IR	
		2b:P1.5	1 (1.9)	0.06	S	1 (IR)
		2b:NST	1 (1.9)	0.25	IR	1 (IR)
			1 (1.9)	0.5	IR	
	15 (n = 1)	15:NST	1 (1.9)	0.06	S	
	NT (n = 3)	NT:P1.2,5	1 (1.9)	0.06	S	
		NT:P1.5	1 (1.9)	0.06	S	
		NT:NST	1 (1.9)	0.06	S	
W135 (n = 3)	2a (n = 3)	2a:P1.2,5	2 (66.7)	0.06	S	4 (3S, 1IR)
			1 (33.3)	0.125	IR	

[a]NT, non-serotypeable; NST, non-serosubtypeable.
[b]S, susceptible; IR, intermediate resistance.
[c]Phenotypes that already existed in a strain collection isolated in nine Portuguese hospitals from January 1995 to December 1999 (n = 54) (8). Phenotypes present in the earlier collection not found in our study (unpub. data) are: B:2b:P1.5, B:4:P1.7, B:15:P1.9, B:15:P1.13, B:15:NST, B:NT:P1.14, B:NT:P1.16, C:2a:P1.14, C:2a:NST, C:4:P1.12; W135:NT:NST (n = 1 for each phenotype).

penicillin, and C:2b:P1.2,5 strains had the highest MICs of penicillin (all isolates with MIC of 0.5 µg/mL and 95% with MIC of 0.25 µg/mL). In Spain in the 1980s, types 4:P1.15 (serogroup B) and 2b (serogroup C) were also found in the main meningococci with intermediate resistance to penicillin (14).

In conclusion, continued serogrouping of meningococcal strains would be valuable because possible antigen variations caused by capsular switching (15) can occur after the period of this study, especially because of the voluntary vaccination program available in Portugal. Meningococci use this mechanism to escape control by vaccines or the natural immune protection. Consequently, the pattern of phenotypes could be subject to major changes, and adjustment to new circumstances will be needed. Antigenic characterization has contributed to the

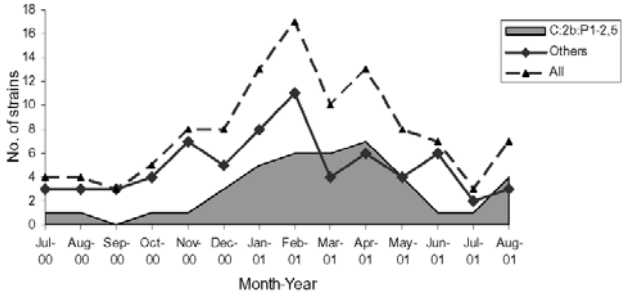

Figure. Distribution of *Neisseria meningitidis* collected in Portugal from July 2000 to August 2001 and phenotype C:2b:P1.2,5.

understanding of meningococcal diversity and spread of meningococcal disease. Trends were detected epidemiologically, and serotyping provided further information, which can also contribute to establishing strategies for developing a universal serogroup B vaccine. Molecular typing of *N. meningitidis* is also needed in Portugal to follow evolutionary changes in the bacteria and to elucidate clonal relationships between isolates.

Our results also demonstrate the importance of monitoring susceptibility to antimicrobial drugs and antigenic characteristics of meningococci; in Portugal, the prevalent phenotype C:2b:P1.2,5 is of particular concern.

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Immunofluorescence Assay for Serologic Diagnosis of SARS

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We evaluated an indirect immunofluorescence assay based on virus-infected cells for detecting anti–severe acute respiratory syndrome-associated coronavirus (SARS-CoV) immunoglobulin (Ig) G antibody. All confirmed SARS cases demonstrated seroconversion or fourfold rise in IgG antibody titer; no control was positive. Sensitivity and specificity of this assay were both 100%. Immunofluorescence assay can ascertain the status of SARS-CoV infection.

On March 12, 2003, the World Health Organization (WHO) issued a global alert on outbreaks of atypical pneumonia (1). Cases were observed in Vietnam, Hong Kong, Singapore, and Toronto. As of June 2003, a total of 29 countries had been affected (2). WHO refers to this highly infectious disease as severe acute respiratory syndrome (SARS) and has formulated case definitions for surveillance (3). The virus causing SARS was identified in late March (4–6). The full genome of a few strains of the SARS-associated coronavirus (SARS-CoV) was soon available; it was confirmed to be a novel virus phylogenetically distinct from previous known coronaviruses (7,8). Since the discovery of SARS-CoV, laboratory diagnosis for the infection has become an important part of patient management, contact tracing, and epidemiologic study. In general, serology is the mainstay for ascertaining viral infection status. We report the evaluation of a first-generation assay based on the indirect immunofluorescence technique for detecting anti-SARS-CoV immunoglobulin (Ig) G antibody.

The Study

We conducted this study at the teaching hospital of the Chinese University of Hong Kong, Prince of Wales Hospital, where a major outbreak of SARS had occurred (9). Patients admitted with clinical features suggestive of SARS were investigated for SARS-CoV infection by a combination of methods including direct detection of viral RNA by reverse-transcription polymerase chain reaction (RT-PCR) using primers COR1/COR2 (10), virus isolation using African green monkey kidney (Vero) cells, and serology. All RT-PCR–positive results were confirmed by repeat testing from the original sample; isolation positive results were confirmed by detection of SARS-CoV RNA from culture supernatant by RT-PCR.

True SARS Cases

For the purpose of study analysis, a patient was defined as a true SARS case when he or she had the following two conditions: 1) fulfilled the WHO criteria of a probable case of SARS (11), and 2) had one or more specimens positive for SARS-CoV by RT-PCR, isolation, or both. From March to May 2003, we identified 128 patients who fulfilled our definition of a true SARS case. Sixteen of them died before a convalescent-phase blood sample could be collected; 9 received convalescent-phase plasma therapy. These 25 cases were excluded. As a result, 103 true SARS cases were analyzed. Three were pediatric patients of ages 5, 11, and 16 years. Eighty-six were adults from 21 to 64 years of age (mean 35.7, SD 11.3), 60.5% were female. The remaining 14 were elderly patients 66 to 89 years (mean 75.6, SD 7.8), 50.0% female. Pneumonia developed in all these patients; five required intensive care and eventually recovered; four died of the infection.

Non-SARS Controls

Patients admitted to the Prince of Wales Hospital during 2000 for respiratory tract infections or febrile illnesses were used as non-SARS controls. The convalescent-phase serum samples that had been collected from these patients for viral and atypical pneumonia serologic screening were retrieved for this study. This control group consisted of 540 patients; 126 were pediatric patients 6 months to 15 years of age (mean 7.4, SD 3.1); 40.0% were girls. Of the 308 adults ages 16–65 years (mean 45.6, SD 10.3); 35.3% were female. For the 106 controls 65–86 years of age (mean 73.2, SD 3.7), 65.0% were female. Overall, 16.3% of this control group were confirmed to have infections with respiratory viruses or atypical pathogens.

In addition to hospitalized patients, a healthy group was included as non-SARS controls. This group comprised 635 medical students 19–31 years of age (mean 23.5, SD 2.2); 41.9% were female. Their blood samples, which had been submitted for pre–varicella-zoster virus vaccination screening in 2000, were retrieved for this study.

Antibody Detection

Anti-SARS-CoV IgG antibody was detected by the indirect immunofluorescence technique. Vero cell monolayer at 90% confluence was inoculated with SARS-CoV. The coronavirus stock used was the third passage of an iso-

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late grown from a SARS patient. The full genome sequence of this isolate has been published (GenBank accession no. AY278554). Infected cells were harvested when cytopathic effect was observed on 70% of the cell monolayer. With our laboratory conditions, this event occurred consistently at 96 to 100 hours after virus inoculation. Infected cells were mixed with noninfected Vero cells at a ratio of 1 to 1. After being washed three times with phosphate-buffered saline (PBS), cells were spotted onto 12-well, Teflon-coated glass microscope slides. The slides were allowed to air-dry and then fixed for 10 minutes with 100% pre-chilled acetone, and stored at –70°C until use.

Serum samples were heat-inactivated at 56°C for 30 minutes and then diluted in PBS. An aliquot of 25-μL diluted serum sample was placed on a coated well and incubated at 37°C for 30 minutes in a moist chamber. After being washed three times with PBS, a fluorescein isothiocyanate–conjugated rabbit anti-human IgG antibody (Dako, Denmark) was added at a dilution of 1 to 40, and incubated for 30 minutes at 37°C. In each test run, a positive control serum with known titer was tested in twofold serial dilutions to guard the sensitivity, and results were crosschecked by two experienced technicians.

Our diagnostic approach was to perform a screening test at a dilution of 1 to 40 for serum samples collected at $\geq$10 days after the onset of illness. Upon special circumstances, testing might be performed on earlier samples. When the screening result was positive, a follow-up test at twofold serial dilutions starting from 1 to 40 was performed together with the corresponding acute-phase serum sample. On the other hand, if the screening result was negative or showed nonspecific fluorescent signals, a follow-up sample was collected for repeat testing. In addition, when a titer of 1:40 was obtained on the second sample, a third sample was collected for repeat testing. A seroconversion or fourfold rise in antibody titer was regarded as serologic evidence of recent SARS-CoV infection.

The serologic data of the true SARS cases used for this analysis were based on the results obtained from our routine test runs. During the outbreak, our laboratory-performed screening test are conducted on every alternate day, and follow-up titration tests occur the next day. The samples included in each test run were based on clinicians' requests containing a variable proportion of cases that turned out to be non-SARS; the technicians did not know the results of other SARS investigations for the testing samples. For the non-SARS controls, each test run contained 50 testing samples mixed with 5 known positive controls and was tested in a blind fashion.

Conclusions

A total of 212 serum samples from the 103 true SARS cases were tested for anti–SARS IgG antibody. Four samples (1.9%) showed fluorescent signals from all cells fixed on the slide. Since we had mixed infected cells with an equal amount of noninfected cells, we expected to observe genuine positive signals from approximately 50% of cells fixed on the slide. Therefore, these four samples were regarded as nonspecific. Follow-up samples were obtained from these patients, and all tested positive. Overall, 94 (91.3%) cases showed seroconversion, and 9 (8.7%) showed a fourfold rise in antibody titer. The positive rate and antibody titer with respect to the time of specimen collection are shown in the Table. We detected the earliest seroconversion on day 6 after the onset of fever. The antibody-positive rates for samples collected during days 5–10, 11–15, and 16–20 after the onset of fever were 34.3%, 78.3%, and 97.7%, respectively.

Of the 1,175 samples obtained from the non-SARS control groups, 24 (2.0%) showed fluorescent signals from all cells fixed on the slide. These samples were regarded as nonspecific. The remaining 1,151 samples were negative for anti–SARS IgG antibody.

Serologic diagnosis remains an indispensable means for confirming viral infection status. Antibody assays based on virus-infected cells or whole viral lysate might produce cross-reactivity between infections because of closely related viruses. As common cold–associated coronavirus infections are highly prevalent, the specificity of whole virus-based assays for the diagnosis of SARS-CoV infection is a concern. Our results indicated that an infected cell-based indirect immunofluorescence test for anti-SARS IgG antibody provided a sensitivity and specificity of 100%. However, this immunofluorescence test is relatively labor intensive. Experienced technicians are

Table. Anti-SARS-CoV IgG positive rate and titer according to time of blood sample collection[a]

Time of sample collection after onset of fever	No. of samples tested	No. (%) of samples with anti–SARS IgG antibody detected	Anti-SARS IgG antibody titer, range (mode)
1–5 days	64	0	–
6–10 days	35	12 (34.3)	40–320 (160)
11–15 days	23	18 (78.3)	40–640 (320)
16–20 days	43	42 (97.7)	40–2,560 (640)
21–37[b] days	47	47 (100)	80–5,120 (640)

[a]SARS, severe acute respiratory syndrome; CoV, coronavirus; IgG, immunoglobulin G; –, not applicable.
[b]Median collection time: 22 days; interquartile range: 4 days.

required to examine the results, in particular to differentiate nonspecific signals from positive results. These properties make the test not ideal for large-scale studies. Nevertheless, its high sensitivity and specificity make the test applicable to ascertain infection status and to serve as a reference for assessing the performance of high-throughput second-generation assays such as enzyme immunoassay. The immunofluorescence test can also be used as a confirmatory assay for samples reactive to screening assays. Further development of more feasible assays with high throughput and performance should be pursued. Evaluation of the role of other classes of anti–SARS-CoV antibodies in the diagnosis of SARS-CoV infection is needed.

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Tick-borne Encephalitis in Southern Norway

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The first five cases of human tick-borne encephalitis in Norway were reported from Tromöya, in Aust-Agder County. Serum specimens from 317 dogs in the same geographic area were collected. An enzyme immunoassay demonstrated antibody to human tick-borne encephalitis virus in 52 (16.4%) of the dogs, which supports the notion of an emerging disease.

First described in humans in Austria (1), tick-borne encephalitis (TBE) is rapidly becoming a growing public health problem in Europe (2). Although observations indicated antibody presence in humans in southern Norway (3), this country has been absent from maps visualizing TBE-endemic areas. This situation may be changing. The first case of clinically manifest TBE in humans in Norway was reported in 1998 (4). Four additional cases were described from 1998 to 2001; all five cases were from Tromøya in Aust-Agder County of southern Norway (5). Infected dogs indicate that TBE virus (TBEV) is present in different geographic areas. The first case of TBE in dogs was reported by Lindblad in Sweden (6), and later by others in Central Europe (7). We investigated and found TBEV immunoglobulin (Ig) G in dogs in southern Norway, an area where this virus was not previously considered endemic.

The Study

From 1992 to 2000, we collected serum samples from 317 (65 breeds, 146 male, 171 female) dogs seen at a veterinary clinic in Arendal, in southern Norway. The laboratory received 436 serum specimens. In case of multiple specimens from one dog, collected during several months or years, we controlled the results for possible changes in antibody levels.

We used two different enzyme-linked immunosorbent assay (ELISA) techniques. The presence and level of IgG antibodies to TBEV were tested by an enzyme immunoassay for the detection of IgG antibodies to TBEV (Enzygnost Anti-TBE virus IgG, Dade Behring Marburg GmbH, Marburg, Germany). Antibody levels ≥1:100 were considered positive. Controls were obtained from the laboratory InVitro (InVitro, Vienna, Austria). IgG to TBEV was detected by a specific sheep, anti-dog, heavy and light chain IgG antibody (A40-105P-7, Bethyl Laboratories, Montgomery, TX) in a dilution of 1:20,000. Positive specimens were confirmed by a second ELISA (Baxter-Immuno, Orth, Austria), as previously described (7). In this assay, titers ≥100 were considered to be positive.

Results

A total of 52 (16.4%) of 317 dogs had IgG antibodies to TBEV; 40 (12.6%) had IgG antibody titers to TBEV ≥450, while 12 dogs (3.8%) had moderate levels (≥100–<450) (Table 1). Positive serum specimens, including samples with 11 to <100 U in the enzymeimmunoassay (EIA)-E test, were confirmed with the Baxter-Immuno (B-I) test (Table 2). The confirmatory test included five extra serum samples in instances where such blood samples were drawn; thus the number of positive specimens to be confirmed was 57.

We could not confirm one result (no. 287) with 116 U in the Enzygnost (EIA-E) by the Immuno ELISA. Of the low-positive specimens in the Enzygnost (<100 U), only four specimens had low-positive results in the B-I ELISA; all others were negative. Four low-positive EIA-E specimens gave positive results in the B-I test. On the other hand, 9 low-positive specimens in the EIA-E (20–37 U) were negative by the B-I test.

The codes were not broken until after the experiments were performed. Thus serum specimens sampled and coded at different times were in some cases collected from

Table 1. Results of serologic examination of canine serum specimens for antibodies to tick-borne encephalitis virus

U	N
<100	265
≥100	52
Total	317

Table 2. Distribution of positive canine serum specimens[a]

EIA-E U ≥450	N	EIA-E U ≥100–<450	N	EIA-E U ≥11–<100	N
B-I titer		B-I titer		B-I titer	
3,200	1	3,200	0	3,200	0
1,600	5	1,600	0	1,600	0
800	5	800	0	800	0
400	18	400	4	400	0
200	12	200	2	200	1
100	3	100	3	100	3
Total	44		9		4

[a]Positive by enzyme immunoassay E-test (EIA-E) and confirmed by a second test (enzyme immunoassay Baxter-Immuno [EIA-BI]). In five cases, we had two or more serum specimens with high positive results. All these samples were tested by both enzyme-linked immunosorbent assay techniques.

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Table 3. Distribution of antibodies according to size of dog

Breed	N positive serum specimens ≥100 U	N negative serum specimens ≤100 U	Total	% positive
Alsatian wolf dog	7	33	40	21.2
Bernese Mountain dog	1	7	8	14.3
Bouvier	1	6	7	16.7
Finnish dog	1	4	5	25.0
Flat Coated Retriever	5	11	16	45.4
Golden Retriever	4	21	25	19.0
Hovawart	1	2	3	33.3
Labrador Retriever	5	16	21	31.2
Newfoundland dog	1	8	9	12.5
Giant Schnauzer	3	3	6	50.0
Rottweiler	2	7	9	28.6
St. Bernard	3	0	3	100.0
Total, large dogs	34	117	151	22.5
Total, other dogs	18	148	166	10.8
Total, all dogs	52	265	317	16.4

a single dog. Nevertheless, high positive antibody levels were reproducible even after several years. In five instances, we had two or more serum specimens from one dog with high positive results at our disposal. All these samples were tested by both ELISA techniques.

Only results of ≥450 U in the Enzygnost test could be registered, which in two instances gave lower results in the new specimens. The Immuno ELISA was in agreement with the Enzygnost in case A, and it showed stable titers in case B. In cases C and D, one could observe an increase in titers by the B-I test. We observed seroconversion in three cases.

The average age of the dogs at the time of blood sampling was 6.6 years (0.5–15). The 52 dogs with ≥100 U were 8.02 years versus dogs with <100 U, which were younger, 6.29 years. The distribution of antibodies according to the size of the dogs is shown in Table 3. A total of 34 (21.8%) of 151 large dogs had antibodies to TBEV ≥100 U versus 18 (10.8%) of 166 small and medium-sized dogs. Large dogs were defined as having a body weight of ≥20 kg. This difference is statistically significant: with odds ratio = 2.39, $\chi^2 = 7.03$, p = 0.008 with Yates' correction. Among dogs with ≥450 U, 25 (62.5%) of 40 were large.

Conclusions

Antibodies to TBEV were detected in 16.4% of dogs in Aust-Agder County of southern Norway. This finding indicates that TBEV is present in this geographic region. Although the first human cases prove the existence of TBEV in southern Norway, the levels of seropositivity in dogs were still unanticipated in a region where TBE has previously not been seen.

TBE in dogs has been reported from several European countries (7), and the number of cases is growing. Searching for antibodies to TBEV in our canine population would be useful since dogs are suitable serologic indicators of TBEV in a geographic area, and canine serum has been used to reveal natural epidemic foci. Our data support the recent findings of human TBE cases in Norway and the notion of an emerging disease, especially because the serum samples were collected from the same geographic area where the first human cases were described. The changing epidemiologic situation suggests that better monitoring of TBE is needed in Norway.

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Endemic Carbapenem-resistant *Pseudomonas aeruginosa* with Acquired Metallo-β-lactamase Determinants in European Hospital

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Acquired metallo-β-lactamases (MBLs) can confer broad-spectrum β-lactam resistance (including carbapenems) not reversible by conventional β-lactamase inhibitors and are emerging resistance determinants of remarkable clinical importance. In 2001, multidrug-resistant *Pseudomonas aeruginosa* carrying bla_{VIM} MBL genes were found to be widespread (approximately 20% of all *P. aeruginosa* isolates and 70% of the carbapenem-resistant isolates) at Trieste University Hospital. Clonal diversity and heterogeneity of resistance determinants (either bla_{VIM-1}-like or bla_{VIM-2}-like) were detected among MBL producers. This evidence is the first that acquired MBLs can rapidly emerge and establish a condition of endemicity in certain epidemiologic settings.

Bacterial pathogens bearing acquired metallo-β-lactamase (MBL) genes exhibit a broad-spectrum resistance to β-lactams that is not reversible by serine-β-lactamase inhibitors (e.g., clavulanate and penicillanic acid sulphones), since MBLs are capable of hydrolyzing most β-lactams and are not susceptible to inhibitors. Because of the efficient carbapenemase activity of these enzymes, the resistance profile of MBL producers notably includes also carbapenems, which are the β-lactams with the broadest spectrum of activity and are among the "last resort" drugs for the treatment of gram-negative nosocomial infections. In addition, MBL producers most often exhibit resistant phenotype to additional classes of drugs since they originate nosocomially and acquired MBL genes typically cluster with other drug resistance determinants in the variable region of multi-resistance integrons (1–3). For these reasons, infections caused by MBL producers can pose a substantial challenge for antimicrobial chemotherapy.

The IMP and VIM enzymes are the most common types of acquired MBLs (2,3). The IMP enzymes were first reported in Japan (4), while the VIM enzymes were first reported in Europe (5), but both types of enzymes are now emerging in Asia, Europe, and the Americas as acquired resistance determinants in nosocomial isolates of *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Acinetobacter* spp. and other nonfastidious, gram-negative nonfermenters (3). The VIM-1 enzyme is 90% amino acid homologous with the VIM-2 variant and <40% amino acid homologous with the IMP enzymes (3). Both types of resistance genes are carried on mobile gene cassettes inserted into plasmid- or chromosomal-borne integrons, a location that eventually facilitates horizontal spreading among different strains (3).

Thus far, strains with acquired MBLs have usually been reported sporadically or as causing small nosocomial outbreaks (4,6–8), while longitudinal surveys have demonstrated, at most, a low-level endemicity of MBL producers in hospitals where similar strains have been detected (9,10). One major hospital outbreak, caused by an MBL-producing *P. aeruginosa* clone, was recently reported in Greece (11). We describe the emergence of high-level-endemicity for MBL-producing *P. aeruginosa*, which has recently occurred in a hospital setting of southern Europe.

The Survey

In the University Hospital of Trieste (northern Italy, at the border with Slovenia), clinical isolates of *P. aeruginosa* producing VIM-type MBLs were detected sporadically, for the first time, in 1999 (12). In 2001, a significant increase in the prevalence of imipenem-resistant *P. aeruginosa* isolates was observed at the Laboratory of Clinical Microbiology of that hospital (29%, vs. 19% in 2000 and 21% in 1999, respectively; $p < 0.001$ according to the χ^2 test; statistical analyses were conducted with Epi Info statistical software, version 6.03, Centers for Disease Control and Prevention, Atlanta, GA).

Of the 444 nonreplicate imipenem-resistant *P. aeruginosa* isolates collected in 2001, a total of 89 were randomly selected and analyzed for acquired MBL genes of the bla_{IMP} and bla_{VIM} types in dot-blot hybridization experiments carried out with purified genomic DNA spotted (0.5 μg per spot) on positively charged nylon membranes (ZetaProbe, Bio-Rad, Hercules, CA) with digoxygenin-labeled DNA probes. The probes were polymerase chain

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reaction amplicons containing internal fragments of the bla_{IMP-1} (754–1,114 nt, EMBL/GenBank database entry S71932) or of the bla_{VIM-1} gene (3,366–3,888 nt, EMBL/GenBank database entry Y18050), respectively obtained using primers IMP-DIA (forward, 5′-GGAATAGAGTGGCTTAATTCTC; reverse, 5′-GTGATGCGTCYCCAAYTTCACT) and VIM-DIA (forward, 5′-CAGA TTGCCGATGGTGTTTGG; reverse, 5′-AGGTGGGCCATTCAGCCAGA) as described previously (13). Hybridization was carried out under conditions that allowed recognition, by each probe, of different allelic variants of the corresponding MBL determinant. None of the imipenem-resistant isolates were recognized by the bla_{IMP} probe, while 64 (72%) were recognized by the bla_{VIM} probe. In the 64 bla_{VIM}-positive isolates, the nature of the determinant was further investigated by analysis of the *Rsa*I restriction fragment length polymorphism of the gene region amplified by the VIM-DIA primers as described previously. With this approach, the determinant was identified as bla_{VIM-1}-like in 54 isolates (84%), and as bla_{VIM-2}-like in the remaining 10 isolates (16%).

The sources of the 64 bla_{VIM}-positive isolates were 52 inpatients from 15 different wards (including 10 medical wards, 4 surgical wards, and an intensive care unit), 5 patients from 4 different long-term care facilities for elderly persons, and 7 outpatients (Table 1). The degree of genomic relatedness of these isolates was investigated by Random Amplification of Polymorphic DNA (RAPD) (14) and by Amplified Fragment Length Polymorphism (AFLP) (15). Electrophoretic profiles generated by the techniques described earlier were compared by the GelComparII software (Applied Maths, Kortrijk, Belgium). Consistent results were obtained with both typing methods. Isolates sharing a Dice similarity coefficient >0.88 comparing their RAPD-profiles were assigned to the same cluster. Results of molecular typing indicated that most bla_{VIM}-positive isolates (61 [95%]) belonged to either of two clusters, indicated as cluster A and B respectively, while the remaining three isolates were unrelated with those clusters and also among each other (Figure). Cluster A included 53 isolates, all containing bla_{VIM-1}-like determinants. They were widely distributed in the hospital (15 wards), and were also found in three long-term care facilities and in six outpatients. Cluster B included eight isolates, all containing bla_{VIM-2}-like determinants. The isolates were from four wards where isolates of cluster A had also been detected. Of the three sporadic isolates, one (carrying a bla_{VIM-2}-like gene) was from a ward where isolates of clusters A and B had also been detected, the second (also carrying a bla_{VIM-2}-like gene) was from a long-term care facility different from those yielding isolates of cluster A, and the third (carrying a bla_{VIM-1}-like gene) was from an outpatient (Table 1). Genotyping of the 25 bla_{VIM}-negative isolates indicated that 5 belonged in cluster A, 1 in cluster B, while the remaining 19 were unrelated to the VIM producers and were overall distributed among 6 different genotypes (Table 1).

Imipenem MICs for the bla_{VIM}-positive isolates were always ≥64 μg/mL (range 64–512 μg/mL), while being always <64 μg/mL for the hybridization-negative isolates. Most of the bla_{VIM}-positive isolates (49 of 64 [76%]) exhibited a multidrug-resistant phenotype including all the tested drugs (imipenem, meropenem, ceftazidime, piperacillin, aztreonam, amikacin, gentamicin, tobramycin, and ciprofloxacin), except polymixin B. On the other hand, this virtually panresistant phenotype was observed in 7 (28%) of 25 bla_{VIM}-negative isolates (Table 2).

Conclusions

Our findings are of concern since they demonstrate that acquired MBLs can rapidly emerge and become a major cause of broad-spectrum β-lactam resistance among noso-

Table 1. Genetic relatedness, presence of MBL determinants, and distribution of the 89 imipenem-resistant *Pseudomonas aeruginosa* isolates[a]

No. of isolates	RAPD–AFLP genotypes[b]	bla_{VIM} allele	Hospital wards (patients)	Long-term care facilities (patients)	Outpatients
bla_{VIM}-positive					
53	A	bla_{VIM-1}-like	15 (43)	3 (4)	6
8	B	bla_{VIM-2}-like	4[c] (8)	-	-
1	C	bla_{VIM-2}-like	1[d] (1)	-	-
1	D	bla_{VIM-2}-like	-	1 (1)	-
1	E	bla_{VIM-1}-like	-	-	1
bla_{VIM}-negative					
5	A	None	2 (3)	-	2
1	B	None	1 (1)	-	-
19	F-G-H-I-J-K[e]	None	8 (16)	1 (1)	2

[a] MBL, metallo-β-lactamase.
[b]RAPD–AFLP, Random Amplification of Polymorphic DNA–Amplified Fragment Length Polymorphism. Results obtained with the two genotyping techniques were always consistent with each other.
[c]In these wards isolates of cluster A were also detected.
[d]In this ward isolates of clusters A and B were also detected.
[e]Genotypes F to K included a number of isolates ranging from 1 to 7.

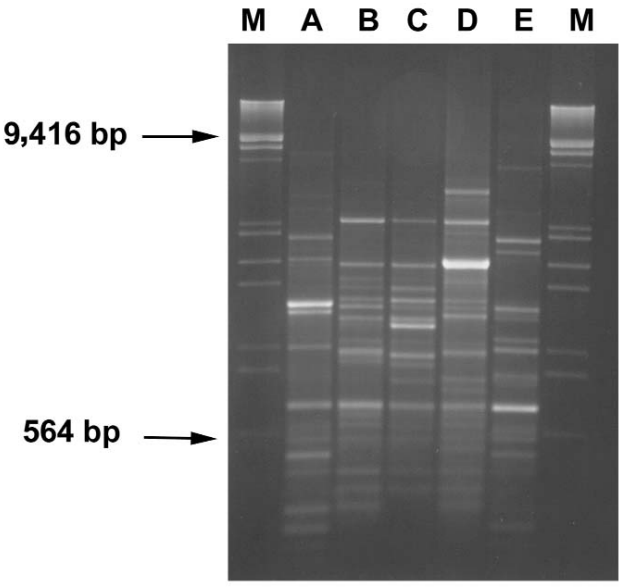

Figure. RAPD profiles of *blaVIM* positive strains. Amplification products (8 μL) obtained with primer 208 (5'-ACGGCCGACC-3') (14) were run on 2% agarose gel. Lanes A-E: RAPD-types as indicated in Table 1. Lanes M: DNA digested with *Eco*RI and *Hind*III.

comial pathogens. In our setting bla_{VIM}-positive *P. aeruginosa* isolates, which were sporadically detected for the first time in 1999 (12), represented approximately 20% of all *P. aeruginosa* isolates and 70% of the carbapenem-resistant *P. aeruginosa* isolates, respectively, during 2001. These figures exceed those reported for MBL producers from other settings (7,9,10). As an additional matter of concern, the bla_{VIM}-positive isolates were significantly more resistant than the bla_{VIM}-negative isolates to non-β-lactam antimicrobial agents as well.

In this survey, the bla_{VIM}-positive isolates were detected on a regular basis during the year and appeared to be widely distributed in the hospital and even outside of it. Molecular characterization showed the simultaneous circulation of different bla_{VIM} alleles (either bla_{VIM1}-like or bla_{VIM-2}-like) in multiple *P. aeruginosa* clones. Overall, these findings suggest that bla_{VIM} determinants have rapidly established a condition of high-level endemicity in this area. To the best of our knowledge, this study is the first in which a similar condition has been reported. Even the large outbreak reported in Greece was caused by a single clone and was apparently confined to the hospital wards (11). The finding of bla_{VIM}-negative *P. aeruginosa* isolates showing the same genotype as that of the two major clusters of bla_{VIM}-positive strains suggests a likely acquisition of the MBL determinants by strains already endemic in this area, followed by clonal expansion of the bla_{VIM}-positive strains.

The possibility that spreading transferable MBL genes among nosocomial gram-negative pathogens could emerge as a major problem in the clinical setting underscores the need for systematic surveillance of these resistance determinants. Considering that MBL producers were also isolated from outpatients and from long-term care facility patients, even if all of them showed at least one hospital treatment during the 6 months before, surveillance should not be restricted to nosocomial isolates but should also include isolates from community-acquired infections.

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Table 2. Antimicrobial susceptibility of the 89 imipenem-resistant *Pseudomonas aeruginosa* isolates[a]

Drug resistance profile[b]									bla_{VIM} status		
									bla_{VIM-1} (n = 54) (%)	bla_{VIM-2} (n = 10) (%)	bla_{VIM}-negative (n = 25) (%)
Imi	Mem	Caz	Pip	Atm	Ak	Gm	Tob	Cip	39 (72)	10 (100)	7 (28)
Imi	Mem	Caz	Pip	Atm		Gm	Tob	Cip	11 (20)	-	6 (24)
Imi	Mem	Caz	Pip			Gm	Tob	Cip	1 (2)	-	1 (4)
Imi	Mem	Caz	Pip		Ak	Gm	Tob	Cip	2 (4)	-	-
Imi	Mem	Caz			Ak	Gm	Tob	Cip	1 (2)	-	-
Other[c]									-	-	11 (44)

[a]All isolates were susceptible to polymixin B. The percentage of isolates resistant to all the tested drugs (except polymixin B) was significantly higher among bla_{VIM}-positive isolates (76% vs. 28%; $p < 0.001$, according to the χ^2 test).
[b]Imi, imipenem; Mem, meropenem; Caz, ceftazidime; Pip, piperacillin; Atm, aztreonam; Ak, amikacin; Gm, gentamicin; Tob, tobramycin; Cip, ciprofloxacin.
[c]Strains resistant to fewer than 5 antibiotics.

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Mycobacterium bovis Infection, United Kingdom

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We describe the first documented spillover of bovine tuberculosis from animals into the human population of the United Kingdom since the resurgence of the disease in cattle in the country. This finding suggests that there may be a small risk for transmission to humans, making continued vigilance particularly necessary.

In the past, *Mycobacterium bovis* was a major source of tuberculosis in humans through consumption of unpasteurized milk. Currently, tuberculosis as a result of *M. bovis* infection is comparatively rare, but it remains a cause for concern in persons at high risk, such as abattoir workers (1). *M. bovis* principally affects cattle, but it can cause disease in a range of wild and domesticated animals, for example, badgers, ferrets, cats, deer, and llamoids (2). In U.K. cattle, *M. bovis* infection is now primarily a pulmonary disease, and the main route of transmission is likely to be through aerosol dissemination. Currently, approximately 1% of human tuberculosis cases can be attributed to *M. bovis*; most of those are likely to follow reactivation (3) or to be recent infections contracted abroad. Tuberculosis caused by *M. bovis* in the young is usually a primary infection. We report two human cases in Gloucestershire. One of the cases may have resulted from intrafamilial spread.

Case Studies

Bovine tuberculosis was diagnosed in two siblings—one currently residing, the other residing until recently, on their parents' farm in Gloucestershire— in 1999. A brother and sister ages 20 and 17 years, respectively, they are thought to have the first cases of indigenously acquired bovine tuberculosis caused by *M. bovis* in persons <25 years of age, with no documented history of travel abroad, reported to the Public Health Laboratory Service Communicable Disease Surveillance Centre (CDSC) since 1990.

When seen by her physician in 1999, the 17-year-old girl had a 6-month history of cough, weight loss, and lethargy. Infection with acid- and alcohol-fast bacilli (AAFB) was confirmed by culture of bronchial washings. Her brother had an 18-month history of cough. He was subsequently found to be AAFB-smear positive with pulmonary cavitation (i.e., he had an infectious case). Sputum samples from both case-patients were cultured by the Gloucester Public Health Laboratory, and the cultures were identified as *M. bovis* by the Regional Centre for Mycobacteriology in Cardiff. All human *M. tuberculosis* isolates are subjected to biochemical analysis and pyrazinamide drug susceptibility testing for differentiation of *M. bovis*. *M. bovis* is therefore detected as part of the routine reference service.

Both siblings had lived on the same farm most of their lives. However, the sister had recently moved into her own place at the time of her diagnosis. Both patients had received *Mycobacterium bovis* BCG in secondary school. Both smoked. Neither had knowingly drunk unpasteurized milk. The girl had no cattle contact. Her brother had occasional cattle exposure: he would assist when stock were confined in a cattle crush[1] for veterinary examination and restrained them by holding their nostrils. During this process, he could become covered in bovine mucus and saliva. He also reported contact with feral ferrets.

No disease has been reported in other family members or in social contacts. Results of screening of other family members (mother, father, and another sibling) were unremarkable. Their father had a grade 2 Heaf test result (and a previous history of BCG) (this is equivalent to a Mantoux response of induration of diameter 5 to 14 mm). Their mother had two grade 1 Heaf test results (Mantoux response of 0 to 4-mm induration) and no history of BCG. The other sibling (age 8) had a grade 1 and a grade 2 Heaf test result and no history of BCG. Heaf grades 0 and 1 or a Mantoux response of 0 to 4 mm induration are regarded as negative; those with a grade 2 reaction (or a Mantoux response of induration of diameter 5 to 14 mm following injection of 0.1 mL purified protein derivative 100 U/mL) are positive. Persons with a grade 2 response are hypersensitive to tuberculin protein and are not given BCG vaccination. A strongly positive reaction to tuberculin is

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[1]A cattle crush is a small, often portable, cage used to restrain individual animals. Crushes are often used in the United Kingdom to hold animals for veterinary attention.

demonstrated by a Heaf grade 3 or 4 or a Mantoux response with induration of at least 15-mm diameter.

The farm had previously held a maximum of 25 beef cattle, introduced around 1981. Tuberculosis (*M. bovis*) herd breakdowns[2] had been recorded by the former Ministry of Agriculture, Fisheries and Food. Five cattle (of 7 slaughtered in a herd of 15) had bovine tuberculosis in 1993; they had caseous lymph node lesions and were culture positive. Another three (out of the herd of eight, which were all slaughtered) had similar lesions in 1997. All infected cattle showed lesions typical of *M. bovis* with confirmatory culture obtained; one had prescapular lymph nodes enlarged with caseous changes. The remaining animals were slaughtered as "direct contacts." Tuberculosis breakdowns have been reported in neighboring herds, and the area supports a substantial badger population. After the 1993 cattle breakdowns, five badgers were trapped; four were positive for *M. bovis* on culture. Similarly, in 1997, a single trapped badger was culture positive.

Conclusions

M. bovis from the cases and from cattle on the farm in 1997 were indistinguishable by a combination of restriction fragment length polymorphism (RFLP) analysis using the IS6110 element, spacer oligonucleotide ("spoligotyping"), and variable number tandem repeat (VNTR) analysis (4–6). RFLP analysis using the IS6110 insertion sequence represents the standard criterion for differentiating *M. tuberculosis*, but it is insufficiently discriminating for *M bovis* due to the paucity of IS6110 elements in the genome of this bacterium. Spoligotyping is based on the polymerase chain reaction (PCR) amplification of a polymorphic direct repeat (DR) locus in which the DR elements are interspersed with up to 43 spacer regions (Figure). The typing process relies on the presence or absence of spacers in the amplified DNA, which are detected by hybridization to a series of synthetic spacer oligonucleotides covalently linked to a filter. The presence of hybridized areas is shown by using a chemiluminescent reaction detected on film as a dark band; absence of spacers shows no binding. The sequence is then displayed as a binary bar code, which can be manipulated digitally. Similarly, VNTR analysis uses PCR to amplify a region in which there are tandem repeats at multiple loci. The result is a digital code describing the number of repeat units at each locus (Figure). The spoligotype profile obtained in these cases is one of the most common seen in bovine tuberculosis in the United Kingdom, and caution is needed before one can say unambiguously that strains have been transmitted. Nevertheless, the combination of typing methods, together with supportive epidemiology, provides evidence of exposure to a common source of infection.

M. bovis was characteristically transmitted to humans by ingestion of infected milk. Thus, historically, human *M. bovis* lesions were primarily extrapulmonary or intestinal. Cattle infected with *M. bovis*, by contrast, usually have pulmonary infection, and shedding of *M. bovis* in respiratory secretions has been reported by several workers (7–9). It is suggested that a possible route of badger to cattle transmission is by inhalation of bacilli from grass contaminated with infected badger urine, feces, or sputum (10). Cattle preferentially graze edges of fields, and they may sometimes be forced to graze close to badger latrines and scent-marking areas at the edge of fields. Cattle-to-cattle transmission of *M. bovis* is also likely to be important. Work to date (11) indicates that particular tuberculosis spoligotypes are usually clustered in specific areas, implying that herd breakdowns are localized events originating from a relatively static reservoir. In many instances, cattle and badgers have been found to share similar spoligotypes (11), but further sampling of badgers, cattle, and other wildlife is required to identify which species can share the infection. Current Department for Environment Food and Rural Affairs research is aimed at establishing the epidemiology and pathogenesis of *M. bovis* and the possible pathways of interspecies transmission.

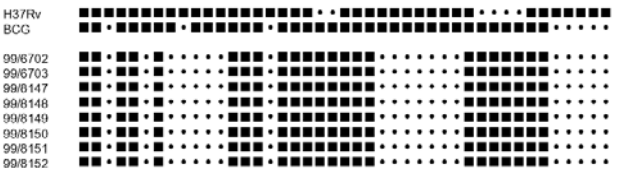

Figure. Spoligotyping profiles for human and cattle cases of bovine tuberculosis. H37Rv and BCG are control strains. 99/6702 and 99/6703 are from the sister and brother, respectively; 99/8147–99/8152 are cattle isolates.

Agricultural workers may acquire the disease by inhaling cough spray from infected cattle. Typical pulmonary tuberculosis then develops, which is what we believe occurred here.

Despite a long history of cattle herd breakdowns on this farm, the family members were not screened until the human cases occurred. Early detection of the disease in the young man before it became infectious might have prevented transmission to his sister and avoided the need for chemoprophylaxis for her infant son. No guidelines were in force at the time. Those subsequently issued (12) advocate screening of human contacts of disease only where pulmonary or udder lesions are detected in cattle. Since the early 1980s, reports of cattle herd breakdowns have steadi-

[2]A tuberculosis breakdown is confirmed when a visible lesion, typical of tuberculosis, is seen by an official veterinary officer in the carcass of a tuberculosis reactor at postmortem or, if *Mycobacterium bovis* is cultured from the set of lymph glands that are routinely collected at postmortem and sent to a state veterinary laboratory for culture.

ly risen, with a more dramatic increase since 1990. The Southwest of England, the West Midlands, and South and West Wales have had recent increases in disease in cattle, and this trend is extending northward to include Derbyshire, Staffordshire, and Shropshire. This incident represents the first documented probable spillover into the human population from animals since the disease's resurgence in cattle, and it suggests there may be a small risk for transmission to humans, even when the bovine case is reported as closed,[3] because of the presence of *M. bovis* in the cattle's respiratory tract (7).

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Dr. Smith is lead clinical scientist in the National Public Health Service for Wales Zoonoses Surveillance Unit, based in Cardiff. His research interests cover a wide range of indigenous and imported, zoonotic, and parasitic infections in the United Kingdom.

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[3]"Closed" refers to a gastrointestinal tract case in which the lymph nodes had not caseated and there was no perceived contact with the outside of the body cavity, as there would be a with a known respiratory (open) infection involving the lungs.

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William Jacob Hays (1830-1875). Prairie Dog Village (1860).
Oil on canvas (25 1/2 x 47 1/2). Collection of National Museum of Wildlife Art, Jackson Hole, WY

Prairie Dog: Cuddly Pet or Trojan Horse?

Abdu F. Azad*

In this issue of Emerging Infectious Diseases, two articles analyze tularemia outbreaks, and one examines the pathologic features of monkeypox in commercially traded prairie dogs. While the prairie dog–associated tularemia outbreak and the first U.S. monkeypox outbreak highlight health risks to humans of the unregulated trade of wild-caught animals, they also raise broader issues. Exotic animal import and export have altered the composition of native fauna and flora throughout the world and have been associated with disease outbreaks. In addition, translocation of animals in some circumstances has provided the opportunity for pathogens to jump species and become established in native animal populations. Examples abound of intentionally or accidentally imported exotic species introducing new pathogens (e.g., rats introducing *Yersinia pestis*, the etiologic agent of plague, to the western United States) and allowing them to become established in native animal populations.

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Avashia et al. (1) and Petersen et al. (2), analyzing the tularemia outbreak in commercially traded prairie dogs, underscore the difficulty of identifying existing infection in wild-caught animals, particularly by commercial collectors and distributors who trap prairie dogs from disease-endemic foci. The outbreak was predictable, considering the conditions in which the animals were kept. In retrospect, the prairie dog–associated monkeypox outbreak highlights the speed with which exotic rodent species, transported worldwide, allow virulent pathogens to jump species and be transmitted to humans. Often, infection with these pathogens is fatal for recipient animals, as is the case in prairie dogs infected with *Y. pestis* or *Francisella tularensis* (the etiologic agent of tularemia). The infected animals can also become "silent" carriers and serve as a new reservoir for the introduced pathogen. The overall outcome of a pathogen's jumping species often remains unpredictable. Guarner et al. (3) investigated the pathologic features of monkeypox in prairie dogs to identify the route of viral transmission to humans. They found that viral transmission could occur through both respiratory

and direct mucocutaneous exposure. In addition, prairie dogs may be an excellent model for assessing monkeypox viral transmission, pathogenesis, and new vaccines and treatments.

Recent incidents involving plague, tularemia, and monkeypox transmission to humans by pet prairie dogs are a wake-up call for better surveillance of wild-caught animals before they are sold internationally and imported into the United States. Relying on visual inspection to select healthy animals is virtually impossible, since wild-caught animals often do not exhibit signs of overt disease and may not appear sick during the early stages of infection with *Y. pestis* or *F. tularensis*. Prairie dog–associated monkeypox and tularemia, as discussed in Avashia et al., Petersen et al., and Guarner et al. (1–3), highlight the need for more research, public education, regulatory guidelines for exotic animal husbandry practices, and possibly a ban on the sale of wild-caught animals. Although the examples in this issue relate to prairie dogs, they may well serve as harbingers of other emerging infections. In introducing seemingly harmless furry friends, the trade of exotic pets brings together species that have never encountered one another in nature, with unpredictable and sometimes tragic results.

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

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Early Defervescence and SARS Recovery

To the Editor: Severe acute respiratory syndrome (SARS) is an emerging disease first recognized November 2002 (1). Previous studies show patients with probable SARS on ribavirin and steroid therapy may experience a biphasic course, with clinical symptoms and changes shown on chest x-rays increasing in the second week of disease (2). We report a patient with probable SARS who had temporary defervescence for 7 days before rapidly progressing to respiratory failure.

The patient was a 54-year-old female nursing aide for a patient with fever and pneumonia who was diagnosed with probable SARS on the basis of the criteria proposed by World Health Organization (WHO) (3). Our patient did not have underlying disease, but fever of 38.6°C developed on May 10, 2003, a total of 3 days after her last contact with the patient she was caring for. Mild myalgia was noted. She was admitted that day with suspected SARS. Initial chest x-ray results were normal. Hemogram showed a normal leukocyte count with mild lymphopenia (absolute lymphocyte count 0.84 x 10^9/L) and a normal platelet count (253 x 10^9/L). Initial serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were 38 U/L and 20 U/L, respectively (normal <35 U/L). Serum creatinine kinase (CK) level was 84 U/L (normal <190 U/L). Serum C-reactive protein (CRP) level was elevated (3.49 mg/dL; normal <0.8 mg/dL). Serum sodium level was normal.

After admission, she received oral ribavirin, 1,000 mg/day. No other antimicrobial agent was administered. Her fever persisted for 2 days, and she became afebrile spontaneously on May 13, 2003. The result of reverse transcription–polymerase chain reaction (RT-PCR) for SARS-associated coronavirus (SARS-CoV) on a throat swab specimen performed on May 10 was negative. All other testing, including blood culture; virus isolation; and serologic tests for SARS-CoV, chlamydiae, mycoplasmas, rickettsiae, influenza virus, parainfluenza virus, adenovirus, respiratory syncytial virus (RSV), and coxsackie virus were also negative. She did not take any nonsteroid antiinflammatory drugs (NSAIDs) or steroids during this period. Her chest x-ray results on May 13 remained normal. Other laboratory testing on May 13 showed resolution of lymphopenia, a lower serum CRP level (2.48 mg/dL), but an elevated lactate dehydrogenase (LDH) level (627 U/L; normal <460 U/L).

During the next 4 days, she remained afebrile. Results of a repeated chest x-ray on May 16 were still normal. The serum CRP level decreased to 2.29 mg/dL. However, borderline leukopenia (4.45 x 10^9/L), borderline thrombocytopenia (167 x 10^9/L), an elevated serum CK level (238 U/L), hyponatremia (128.2 mmol/L), and a progressively elevated serum LDH level (1,138 U/L) were noted. Because she had been afebrile for 5 days, she was discharged on May 17. After discharge, she continued to take ribavirin and be quarantined at home. Unfortunately, fever and rapidly progressive dyspnea developed on May 20. On the same day, chest x-ray showed diffusely increased infiltration over all lung zones. Hemogram showed leukocytosis (leukocyte count 14.03 x 10^9/L). Serum CRP level was elevated to 12.7 mg/dL. Serum sodium level was 129.5 mmol/L. Serum AST level was 135 U/L, serum CK level was 71 U/L, and serum LDH level was 1,719 U/L. All blood cultures and sputum culture for bacteria yielded nothing.

She was intubated on May 21 for respiratory failure. Under the assumption of probable SARS, she was given high-dose methylprednisolone (120 mg/day), and her clinical condition stabilized soon after. The results of RT-PCR for SARS-CoV on a throat swab specimen performed on May 21 were positive. The results of immunofluorescent assays testing for immunoglobulin (Ig) M and IgG against SARS-CoV (performed in the research laboratory at National Taiwan University Hospital on May 21 and 27) were all positive (both IgM titers >1:10; both IgG titers >1:1,000). Sputum culture and Gram stain were both negative. Urine tests were also negative for pneumococcal and *Legionella* antigens. Other serologic tests, including those for chlamydiae, mycoplasmas, rickettsiae, influenza virus, parainfluenza virus, adenovirus, RSV, and coxsackie virus were still negative. The ventilator was removed on June 9.

A previous report pointed out the great variety in the clinical course of SARS (4). We emphasize that even a patient with suspected SARS who became afebrile in the first week and remained so for 7 days without steroid or NSAID treatment still risks deterioration in the second week, as long as some laboratory values remain abnormal. Therefore, defervescence, even up to 7 days, may not be the single indicator for discharging SARS patients. Obtaining normal results for previously abnormal laboratory parameters, including hemogram, CRP, CK, AST, ALT, and LDH levels should be considered when deciding whether a patient can be safely discharged (5).

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Babesiosis in Fairfield County, Connecticut

To the Editor: Human babesiosis, caused by *Babesia microti*, was initially described in the eastern United States in 1970 in a woman vacationing on Nantucket Island, Massachusetts (1). With few exceptions, almost all subsequent cases were recorded from islands in the northeastern United States and Cape Cod, Massachusetts (2), until this illness was diagnosed in 13 patients living in New London County in southeastern Connecticut (3,4). *B. microti* was isolated from white-footed mice, *Peromyscus leucopus*, captured from 1988 to 1990 in the yards of patients. Babesiosis also was diagnosed in persons living in Wisconsin (5) and in New Jersey (6) who acquired the organism locally. The number of cases of babesiosis reported by health departments on their Web sites and by personal communication in Massachusetts, Rhode Island, and New York State, was 330 from 1988 to 2002, 121 from 1994 to 2002, and 542 from 1986 to 2001, respectively. The number of cases reported by the New York City Health Department from 1991 to 2000 was 75.

From 1991 to 2000, babesiosis was diagnosed in 230 persons residing in New London County and adjacent Middlesex County, Connecticut (7). Fifty-three additional cases were reported in five other counties in Connecticut, but epidemiologic data did not indicate that these infections likely were acquired within Connecticut. We now note a new and distinct geographic focus by reporting the isolation of *B. microti* from rodents captured in the yards of two patients in whom babesiosis was diagnosed at Greenwich Hospital in 2002. These patients lived in Greenwich, Connecticut, which is located in Fairfield County in the extreme southwestern part of the state. Neither patient had traveled outside of the immediate area of Greenwich, Connecticut, before onset of illness. We also trapped rodents in the yards of two additional patients in whom babesiosis was diagnosed. These two patients had traveled to Rhode Island shortly before becoming ill. Patients became ill from June 23 to July 7, 2002, and none reported a tick bite.

Attempts to trap small mammals on the properties of the four patients were made on July 22, 23, and 29, 2002. Rodents were captured in Sherman box traps baited with peanut butter and apple. Approximately 0.3 mL of blood was drawn from the heart of each animal into a syringe coated with heparin or uncoated. Blood was kept on ice in the field and then returned to the laboratory. A 3- to 5-week-old male Syrian hamster was injected intraperitoneally with 0.1 mL of each blood sample.

Blood smears were obtained from a drop of blood taken from the tail of each hamster on weeks 3 to 6 after injection. Blood cells were stained with Giemsa and examined for *B. microti* at a magnification of 1,008x. Hamsters were considered uninfected when no parasites were found in 75 fields of stained erythrocytes.

B. microti was isolated from rodents captured at the residences of two of the patients who did not travel outside of the Greenwich area 6 weeks before onset of illness. Blood from two of three white-footed mice and from the two eastern chipmunks, *Tamias striatus*, captured in the yards of the patients, produced infections in injected hamsters. Infections did not develop in hamsters injected with blood from 10 white-footed mice captured at the residences of two patients who visited Wakefield and Charlestown, Rhode Island, shortly before becoming ill.

B. microti is prevalent in rodent populations in Greenwich, Connecticut, and causes human disease. Establishing evidence of *B. microti* in rodents and documenting this protozoan parasite as the cause of human disease in Greenwich are important. Relatively high populations of the vector tick, *Ixodes scapularis*, are present in Greenwich and nearby towns. In 2002, the health departments of Greenwich, Stamford, New Canaan, and Darien submitted 1,671 *I. scapularis* ticks removed from persons to the Connecticut Agricultural Experiment Station for identification and testing for *Borrelia burgdorferi*. Two hundred and thirty cases of Lyme disease were reported from these four towns in 2002 (Connecticut Department of Public Health, unpub. data). With such extensive human exposure to ticks and a relatively large number of Lyme disease cases in these four towns and elsewhere in Fairfield County, the number of cases of babesiosis is likely to increase appreciably in the future.

B. microti has been transmitted through blood transfusion in Connecticut (8). Blood collection agencies in southwestern Connecticut and adjacent Westchester County, New York, should be aware of the possibility that blood donors could be infected with this pathogen. Physicians should also be alert to the possibility that patients could be coinfected with the etiologic agents of Lyme disease or human granulocytic ehrlichiosis. Some patients in whom Lyme disease was diagnosed have been simultaneously infected with *B. microti* (9,10).

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Migratory Thrombophlebitis and Acute Q Fever

To the Editor: Q fever is a worldwide zoonosis particularly common in some cattle- raising countries such as Great Britain, France, Spain, Australia, and Canada (1). Widespread use of diagnostic serologic tests has led to increasing recognition of the disease in many other parts of the world. For example, Q fever has been recently described in Southeast Asia (2).

Acute Q fever usually manifests as a febrile syndrome with hepatic abnormalities or pneumonia; chronic Q fever is commonly associated with endocarditis (3,4). When the disease manifests with an atypical clinical picture, diagnosis becomes difficult.

We describe a patient in whom Trousseau's syndrome developed as the sole manifestation of acute Q fever. To the best of our knowledge, this complication has not been found previously in patients with Q fever.

A 39-year-old, previously healthy man was admitted to the hospital because of fever and subcutaneous painful induration in the legs and arms. Ten days previously, he had fever, generalized aches, and malaise. Forty-eight hours after the onset of these symptoms, he noticed a painful induration and redness along the right calf that spontaneously subsided in a few days. He remained febrile, and additional swollen, painful indurated lesions appeared on his thighs and arms. Pleuritic chest pain developed, and he was taken to the emergency room.

On admission, he was febrile (38.7°C). A tender, indurated cord, 10 cm long, was felt on the greater saphenous vein of the right thigh. The cephalic vein of the left arm and the basilic vein of the right arm were also tender and indurated.

The leukocyte count was 9,800/mm^3; hemoglobin 14.5 g/dL, and platelets 441,000/mm^3. The fibrinogen levels (385 mg/dL) and prothrombin time (12 s, control 10–14 s) were normal. The partial thromboplastin time (40 s, control 26–36 s) was mildly prolonged. Serum aspartate aminotransferase was 75 U/L, serum alanine aminotransferase was 110 UL, and γ-glutamyltranspeptidase was 230 U/L. Antibodies to phospholipids (aPL) measured by enzyme-linked immunosorbent assay were positive (titer of 95 for antibodies of class IgG/titer of 19 for antibodies of class IgM. Results of testing for tumor-associated antigens, such as carcinoembryonic antigen, carcinoma antigen 19-9, prostatic specific anti-

gen, and alpha fetoprotein, were negative. The x-ray films of the chest and computed tomography scan of the thorax and abdomen were normal. The patient was treated with subcutaneous heparin and diclophenac, and fever and migratory thrombophlebitis subsided.

Because the patient had been working with manure several days before his initial symptoms, Q fever serologic testing was requested. The antibody levels measured by complement fixation (CF) against phase II *Coxiella burnetii* antigen was 1:512. By indirect immunofluorescence, the titers of IgM and IgG against phase I and II were 1:64 and 1:512 and 1:256 and >2,048, respectively. Antibody titers against *Mycoplasma*, *Chlamydia*, *Legionella*, enterovirus, and influenza were negative.

Recovery was uneventful and the patient was asymptomatic during a follow-up visit 3 weeks later. Antiphospholipid antibodies were negative. Three months after the acute phase of the infection, new titers of antibodies (CF) against *C. burnetii* were 1:128. Two years after the episode the patient was asymptomatic.

This patient is unique in that he had acute Q fever with migratory thrombophlebitis. A diagnosis of Trousseau's syndrome associated with an occult malignancy was considered on admission, but it was excluded soon. The recent history of exposure to manure was the key for the clinical diagnosis. Although specific anti-coxiella treatment was not given, the patient followed a self-limited course, and both clinical and laboratory abnormalities promptly subsided.

Microscopic vasculitis and thrombosis are commonly found in patients with other rickettsial infections (5), but vascular phenomena must be considered an exceptional event in patients with Q fever. However, thrombophlebitis and pulmonary embolisms have been occasionally reported (6–8). These unusual manifestations have been associated with aPL during the course of acute Q fever (7,8).

Antibodies to phospholipids have been found in 80% of patients in a large series of acute Q fever (9). None of the patients in the study showed thrombotic events or cardiac valve involvement in contrast to patients with lupus or primary aPL syndrome in whom clinical manifestations attributed to aPL developed (9). This observation could be explained by the fact that aPL found in patients with lupus and primary aPL syndrome are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation, which has been characterized as a β2-glycoprotein I (apolipoprotein H). This glycoprotein seems to inhibit the activation of the contact phase system of the intrinsic pathway of blood coagulation (10). On the other hand, apolipoprotein H is not necessary for the aPL activity observed in patients with Q fever and other infectious diseases (10).

According to these studies, the observation of low titers of aPL in the serum of our patient during the acute phase of Q fever must be seen as a finding of uncertain importance not necessarily associated with migratory thrombophlebitis. In short, migratory thrombophlebitis (Trousseau's syndrome) should be added to the ever-growing list of unusual manifestations of Q fever.

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West Nile Poliomyelitis

To the Editor: In the July 2003 article, "Acute Flaccid Paralysis and West Nile Virus Infection" (1), Sejvar et al. reported seven patients with acute onset of asymmetric weakness and areflexia but no sensory abnormalities. The authors also referenced three previously reported cases of West Nile virus (WNV)–associated flaccid paralysis and argued that all of these symptoms could be explained by anterior-horn cell loss. The two

cases of spinal cord pathologic findings published to date demonstrated focal loss of anterior-horn neurons (2,3). We report a case of West Nile poliomyelitis with preserved deep-tendon reflexes, diminished sensory nerve action potentials, and pathologic findings which do not localize to the anterior horn.

An 83-year-old woman sought treatment at the hospital on September 12, 2002, with 3 days of fever, acute confusion, nausea, vomiting, and profound weakness. Computed tomography (CT) of the head was unremarkable. Her examination was notable for dysarthria, tremors, and global weakness with rightsided predominance. Cerebrospinal fluid contained 75 leukocytes/mm^3 (84% neutrophils), glucose 88 mg/dL, and protein 97 mg/dL. On the second hospital day, respiratory failure developed, requiring mechanical ventilation. Electrodiagnostics performed on hospital day 7 demonstrated reduced motor and sensory amplitudes on right median and ulnar nerves, reduced motor amplitudes, and mildly reduced conduction velocities in the right peroneal nerve and right posterior tibial nerves. These findings suggested a predominantly axonal polyneuropathy involving both sensory and motor nerves. No myopathic process was demonstrated. Serum antibodies for WNV (immunoglobulin [Ig] M capture enzyme-linked immunosorbent assay [ELISA] 73.8; IgG capture ELISA 0.738) and cerebrospinal fluid antibody titer (IgM capture ELISA 200.5) were both positive. Weakness, respiratory failure, and preserved deep-tendon reflexes persisted.

On hospital day 15, the patient died after withdrawal of support. Sections from the postmortem medulla and spinal cord were positive for WNV RNA by reverse transcription–polymerase chain reaction testing at the state laboratory. The brain demonstrated characteristic microglial nodules and perivascular lymphocytic infiltrates (4). Spinal cord sections showed leptomeningeal and parenchymal chronic inflammatory infiltrates, often perivascular in location. Patchy cellular infiltration was found throughout the spinal cord, without a predilection for the anterior-horn. Spinal nerve roots demonstrated focal lymphocytic inflammation within the endoneurial compartment. No evidence of a demyelinating process was found.

Our data contradict the thesis proposed by Sejvar et al. that West Nile poliomyelitis is restricted to the anterior-horn (1). The electrodiagnostics showing axonal polyneuropathy and the spinal cord pathologic findings, which did not demonstrate focal loss of anterior-horn neurons, suggest a broader spectrum of the clinical-pathological syndrome of West Nile poliomyelitis than previously described (2,3). Our findings conform to the hypothesis outlined by Jeha et al., which favors a more widespread myelitis (5). We also confirm the findings of preserved deep-tendon reflexes in West Nile poliomyelitis first reported by Glass et al. (6).

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In Reply: The letter by Holman, et al., (1) in this edition of Emerging Infectious Diseases continues to broaden the scope of illness attributable to West Nile virus (WNV) infection. However, we would like to take the opportunity to clarify the conclusions drawn from our article, "AFP and West Nile virus infection" (2). In this article, we reported on seven patients, all of whom had asymmetric weakness without sensory loss, which evolved acutely over the course of several hours. Electrodiagnostic studies of all patients displayed markedly reduced compound motor axon potentials with preserved sensory nerve action potentials; the results were interpreted as consistent with a process primarily localized to the motor axons or, far more likely, given the clinical scenario and pathologic data from WNV-infected animals, the anterior horn cells of the spinal cord. These findings were later substantiated by Li et al. (3). Subsequently, Jeha et al. expanded the scope of illness producing WNV-associated flaccid paralysis by describing several patients with predominant myeloradiculitis (4).

The patient presented by Holman clearly seems to have a condition that, as shown by electrodiagnostics and pathology, localizes to other areas, in addition to spinal anterior horn cells.

However, rather than suggesting that a single case report "contradicts" the anterior horn cell hypothesis, we suggest that WNV-associated flaccid paralysis be viewed as having a spectrum of causes, one of which certainly is poliomyelitis like illness.

The neuropathologic findings in poliomyelitis (due to poliovirus, WNV, or other viruses) are not restricted focally to the anterior horn cells (5). Demonstrating pathologic changes as well as focal anterior horn cell loss in the patient referenced by Holman is in keeping with neuropathologic findings in poliomyelitis. Additionally, the presence of a diffuse axonal polyneuropathy cannot be concluded from Holman's data. Reduced compound motor axon potentials and slowing of conduction velocity could certainly be seen in pathologic conditions affecting anterior horn cells or spinal nerve roots. In addition, reduced sensory nerve action potentials in the median and ulnar nerves alone, without documentation of neuropathy in additional nerves, cannot be used as evidence of a diffuse axonal polyneuropathy, since both of these nerves are commonly prone to entrapment neuropathies. Finally, the context in which the preservation of this patient's reflexes is observed remains unclear. Preserved "normal" deep-tendon reflexes, in the setting of disease that interrupts the reflex arc at any point, are incongruous with established physiologic and clinical concepts.

The seven patients observed by our group clearly had a distinct clinical syndrome with similar clinical findings and electrodiagnostic results. However, as demonstrated by prior reports (3,5–7), multiple mechanisms may lead to WNV-associated flaccid paralysis. In fact, we acknowledge a spectrum of cord, root, and nerve involvement with WVN flaccid paralysis.

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Typhus Group Rickettsiae Antibodies in Rural Mexico

To the Editor: In 2002, the risk of transmission of epidemic typhus in the state of Mexico was assessed by analyzing serum specimens from 393 residents of previous typhogenic areas for immunoglobulin (Ig) G antibodies against *Rickettsia prowazekii*. Louseborne typhus has been a historic scourge in Mexico. In 1576, in a population of 9 million, 2 million deaths were attributed to epidemic typhus (1). These illnesses primarily affected indigenous peoples, who called the illness *cocolixtle* and *matlazahuatl* (2).

In 1951 a national campaign against louseborne typhus was begun by using newly developed technologic approaches, antibiotics, and insecticides, resulting in decreases in the incidence and case-fatality rate. In 1951 >1,000 cases and 737 deaths caused by epidemic typhus were reported in 18 states, and 6,781 localities were identified as at risk (3). By 1965, only 36 cases and no deaths were reported from 12 states with 4,841 localities at risk. Most cases occurred during the cold months of November–April. One third of cases occurred in persons 19–29 years of age with nearly 40% of the deaths in patients aged 15–44 years. In 1979, 10 years had passed without any cases of epidemic typhus reported in Mexico. In the 1980s, three outbreaks of typhus occurred in rural communities, two in Chiapas and one in the state of Mexico (4).

In the state of Mexico, during the period 1893–1907, 7,353 epidemic typhus deaths were reported (annual mortality rate, 52.4/100,000 population); from 1939 to 1943, 1,220 cases were reported with 707 deaths (annual mortality rate, 12.1/100,000 population); and from 1959 to 1963, 64 cases were reported with 14 deaths (annual mortality rate, 0.1/100,000 population) (3). In 1967, Atlacomulco, a county in the state of Mexico that had been free of typhus for 5 years, experienced an outbreak of louseborne typhus associated with a case of Brill-Zinsser disease in a 76-year-old man who had a history of

epidemic typhus. Forty cases were diagnosed and one death occurred (3). The last outbreak in the state of Mexico occurred in 1983 in San Juan Cote in San Felipe del Progreso County, with 22 ill persons and one death (4). Since then, a public health program against *Pediculus humanus corporis* has been conducted in five counties with epidemiologic surveillance for cases of reactivation of latent infection. At the beginning of the 1980s, the rate of infestation with *P. humanus corporis* (*mazahua*) in the indigenous population of the state of Mexico was 100%; in 1988, 58%; and in 1990, 15%. In 1999, indices of infestation between 5% and 12% were detected in this population (5).

In 2002, personnel from the office of the Secretary of Health of the State of Mexico evaluated the risk to the population who lived in previously typhogenic areas to measure the impact of their programs (5). In a cross-sectional study, 393 human serum specimens were analyzed by immunofluorescence assay (IFA) for IgG antibodies against *R. prowazekii,* and a titer of 64 or higher was considered positive (6). Antibodies against *R. prowazekii* were detected in 74 serum samples (seropositivity, 18.8%; 26% for males and 18% for females). The prevalence of antibodies to *R. prowazekii* increased with age; 1–14 years of age (seropositivity 0%), 15–24 years (14%), 25–44 years (17%), 45–64 years (24%), and ≥65 years (48%). Thirty-three (45%) of the serum specimens had a titer of 64, 25 (34%) had a titer of 128, and 16 (22%) had a higher titer. All eight serum specimens with a titer of ≥512 were from persons ≥45 years of age.

The high seroprevalence suggests that this population had extensive exposure to the agent of typhus and its louse vector in the past. The finding of two subjects aged ≥65 years with a titer of 1,024 and four subjects aged ≥45 years with a titer of 512 suggests reactivation of latent *R. prowazekii* with a resulting boost in their antibody titers. These possible cases of Brill-Zinsser disease were likely not severely ill and recovered either with antimicrobial treatment that was effective against *R. prowazekii* or by immune control of the infection without a specific diagnosis.

That IgG antibodies against *R. prowazekii* are absent in young persons suggests that this rickettsia has not been circulating in this population during recent years. The high seroprevalence suggests a human reservoir of latent *R. prowazekii* in this population. The presence of human body lice in this population indicates that there is risk of spreading *R. prowazekii* from an index patient with Brill-Zinsser disease to persons in contact with the patient.

Although the general lack of attention to *R. prowazekii* by scientists, physicians, and public health agencies would lead one to believe that typhus has been eliminated as a public health concern, the recent occurrence of a large epidemic in Burundi (7), infected lice in Rwanda, an outbreak in Russia, a documented case originating in Algeria, and outbreaks every year in Andean Peru (8) indicate that global attention should be directed to surveillance, risk assessment, diagnostic capability, and planning for rapid epidemic control to avoid establishing a large reservoir of latent infection for future epidemics originating from recrudescent typhus in louse-infested populations. Typhus likely poses a similar threat in other parts of the world including the Himalayas, Andes, Afghanistan, and highlands of Africa. Even in industrialized countries, the diagnosis of typhus is likely to be delayed or missed. The potential threat of bioterrorist-disseminated, aerosol-transmitted typhus emphasizes that enhanced attention to and knowledge of typhus are needed throughout the world (9). The requirement concerns not only physician awareness but also wide availability and application of the most appropriate diagnostic laboratory methods.

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Schistosoma haematobium Infection and Buruli Ulcer

To the Editor: Buruli ulcer caused by *Mycobacterium ulcerans* was recognized in 1997 as an emerging public health problem by the World Health Organization (WHO) (1). The disease is found in tropical Africa, the Americas, Australia, and Asia (2). In Benin, severe disease with serious complications is reported with increasing frequency. Buruli ulcer causes serious deformities and disability, particularly since amputating limbs is sometimes required in cases of severe disease such as osteomyelitis (3). Given the effect on the quality of life of those afflicted and the lack of adequate treatment, identifying host risk factors for Buruli ulcer is an important research imperative (2). We investigated one potential risk factor, concurrent infection with *Schistosoma haematobium*. Preliminary data indicate that although *S. haematobium* is not a risk factor for Buruli ulcer, it may be associated with osteomyelitis.

Although Buruli ulcer and schistosomiasis each exist in the absence of the other, close parallels exist between their epidemiology, suggesting that schistosomiasis could be one possible risk factor for Buruli ulcer. Both diseases are associated with the tropical wetlands of west and central Africa. Cases of both schistosomiasis and Buruli ulcer have increased rapidly in these areas since the 1980s, particularly after irrigation and dam construction. Buruli ulcer is most frequent in children <15 years of age; this group typically also has the highest prevalence and intensity of schistosome infections. Schistosomiasis is transmitted through contact with infected water when the cercarial larvae penetrate skin, and increasing evidence exists that *M. ulcerans* proliferates in the bottom mud of stagnant waters and may be harbored by aquatic insects (4).

An immunologic rationale for linking the two diseases has been proposed (5). Briefly, protective immune responses to other mycobacterial diseases are known to depend on a type 1 cellular response, typified by interferon-gamma (IFN-γ). Helminth infections, on the other hand, are classically associated with type 2 responses, typified by interleukin (IL)-4 and IL-5 production. Therefore, a concurrent infection with a bloodborne helminth such as *S. haematobium* may skew the immune response away from a potentially protective type 1 response (5).

A total of 113 patients were recruited from Buruli ulcer treatment centers in Lalo (Couffo Department) and Zagnanado (Zou Department) in Benin. A team of experienced surgeons clinically confirmed all cases of Buruli ulcer. Controls (n = 429) were recruited at random from residents of eight current Buruli ulcer foci in the Couffo Department. Past or current Buruli ulcer patients were excluded from the lottery for controls.

Clinical records reported no case of intestinal schistosomiasis in this area. This finding was confirmed by a preliminary survey of 60 Buruli ulcer patients, which detected no concurrent *S. mansoni* by using the Kato Katz method (6). Diagnosis of *S. haematobium* (urinary schistosomiasis) was performed by filtering three urine samples given on different days. Neither cases nor controls were asked to exercise (as is usual) before giving urine samples because many Buruli ulcer cases were immobile. All patients positive for *S. haematobium* were offered praziquantel treatment.

In the entire participating population, 11.5% (95% confidence interval [CI] 6% to 19%) of Buruli ulcer cases were positive for *S. haematobium*; 11.1% (95% CI 5% to 20%) of cases from Lalo and 12.2% (95% CI 4% to 26%) from Zagnanado were positive. The difference between the two centers was not statistically significant. Of the 429 non-Buruli ulcer controls, 9.5% (95% CI 7% to 13%) were positive for *S. haematobium*. No statistically significant difference between cases and controls was detected. The odds ratio for *S. haematobium* infection in a logistic regression model (which also included age and sex) was 1.3 (95% CI 0.63 to 2.4). Prevalence of *S. haematobium* infection did not significantly differ between controls' residence (data not shown). Power analysis indicates that about 4,000 cases and controls would be required to find a statistically significant difference at this prevalence of schistosomiasis.

Both schistosomiasis and Buruli ulcer are very local in nature; one village can have substantial numbers of cases whereas the next village could have none. *S. haematobium* foci with infection prevalence >50% do exist in Benin but in different settlements from the Buruli ulcer foci. Should a Buruli ulcer focus coincide with a schistosomiasis focus with a higher prevalence of infection, some association between the two diseases could appear.

Detailed clinical information was available for 36 patients tested for *S. haematobium*. In all cases, at least two of four laboratory tests were positive for *M. ulcerans*. These tests were: 1) acid-fast bacilli in a smear stained by the Ziehl-Neelsen technique, 2) positive culture of *M. ulcerans*, 3) histopathologic examination of a tissue specimen, and 4) positive polymerase chain reaction (PCR) for *M. ulcerans* DNA. Five patients had confirmed infection in bone samples, so they were classified as osteomyelitis

patients. Two of these five had concurrent *S. haematobium*, compared to no cases in nonosteomyelitis patients; a Fisher exact test showed this difference to be significant ($p < 0.02$).

These limited and preliminary data are consistent with the relationship between leprosy (caused by a mycobacterium related to the one causing Buruli ulcer, *M. leprae*) and concurrent helminth infections. The severity of leprosy has recently been linked to intestinal helminth infection, whereas the presence or absence of leprosy has not (7). The cytokine environment created by helminth infection may facilitate disease progression to a more severe form, or severe mycobacterial disease and helminth infection may have a common risk factor.

We were unable to furnish evidence of a link between the presence or absence of *S. haematobium* infection and Buruli ulcer, but concurrent infections could influence Buruli ulcer clinical manifestation and disease severity.

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1998 Dengue Hemorrhagic Fever Epidemic in Taiwan

To the Editor: The rapid spreading of dengue viruses has led to increasing incidence rates of dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) worldwide in the past 20 years. The global pandemic of DF and DHF in 1998 was associated with the largest DF epidemics many tropical or subtropical countries had ever experienced (1,2). Here we report the unique epidemiologic characteristics of DF and DHF caused by dengue virus type 3 (DEN-3) in Taiwan, where dengue is not endemic.

The recent epidemics of dengue in Taiwan started when dengue virus type 2 (DEN-2) was first introduced into the southern off-islet of Hsiao-Liu-Chiu in 1981 after an absence of 38 years since World War II. Tainan City in southern Taiwan had not had a dengue epidemic since l942–1943 until three dengue outbreaks occurred there in the last decade. The first outbreak of DEN-1 in 1994 and the second of DEN-2 in 1997 involved few confirmed cases. The third epidemic of dengue, which was attributed to DEN-3, began in October 1998 and continued into January 1999.

From August 1, 1998, to January 31, 1999, physicians in all the hospitals and clinics in Tainan City were required to report any suspected dengue cases who met the criteria of fever (>38°C) and two or more of the following symptoms and signs: headache, retroorbital pain, myalgia, arthralgia, rash, and hemorrhagic manifestations. Patients who met the criteria were invited to participate in the study; informed consent was given by the patients, and plasma or serum samples were collected for laboratory confirmation. When a physician reported a suspected dengue case, a minimum of 100 blood samples would be collected from the patient's neighbors by the Tainan City Health Bureau staff. The blood specimens were transported to the laboratory at the National Institute of Preventive Medicine for confirmation. A confirmed dengue case was required to be positive by either reverse transcription–polymerase reaction (3), or demonstrate seroconversion by dengue-specific immunoglobulin (Ig) M and seronegativity for Japanese encephalitis virus (JEV)-specific IgM by IgM-enzyme-

linked immunosorbent assay (IgM-ELISA) (4). Dengue viruses were isolated in C6/36 cell cultures and identified to serotype with monoclonal antibodies (5). The clinical diagnosis of DHF was based on the World Health Organization's (WHO) criteria that were revised in 1997. Those confirmed dengue cases were classified as primary, secondary, or indeterminate infections, depending on the ratio of dengue-specific IgM/IgG as measured by the capture IgM and IgG ELISA (6).

Of 225 case-patients with suspected dengue, 142 patients, of which 74 (52.5%) were female and 68 (48.2%) were male (62.7%), had their cases confirmed by laboratory diagnosis during the study period. Their ages ranged from 7 to 79 years (mean: 39 years). Of the 23 DHF case-patients meeting the WHO's case definition, 17 were male. The ages of the DHF case-patients ranged from 13 to 73 years, with a mean age of 42 years.

The epidemic began in October and peaked in November. In the Central District, where the earliest and majority of DHF cases occurred (52.2%), the DHF/DF ratio increased with time, from 11% during the first interval, to 20% and 30% during the second and third intervals, respectively. Although this increase was not statistically significant by Fisher's exact test because of the small sample size, similar results were reported in Cuba's DHF epidemic in both 1981 and 1997 (7). Many RNA viruses, such as influenza, increase in virulence through transmission; possible virulence mechanisms of dengue viruses are now under investigation. In other words, the duration of epidemic has to be as short as possible to avoid the emergence of DHF cases in that region.

In our study, 88 dengue cases (62%) were classified as primary infections, 32 (22.5%) as secondary infections, and 22 (15.5%) as undetermined because of the lack of paired acute- and convalescent-phase samples. DHF cases showed no significant association with secondary infections (odds ratio = 1.92 [95% CI 0.64 to 5.76], p = 0.19). Because the last documented transmission of dengue viruses in Taiwan occurred during the island-wide dengue epidemic in 1942–43, both DF and DHF cases were stratified into two birth cohort groups. Of DF case-patients born after 1943, 94% (83/88) had primary infection, and 11 (92%) of 12 DHF case-patients had primary infection. By contrast, 84% (27/32) of all the dengue case-patients born before and during 1943 had secondary infection, and 7 (78%) of 9 DHF case-patients had secondary infections. After the cohorts were stratified by age, DHF cases were not associated with secondary infection (Mantel-Haenszel) weighted odds ratio: 0.84 [95% CI: 0.11 to 5.62]) (p = 0.6). Therefore, age was not a confounding factor or effect modifier for DHF case-patients in Tainan's 1998 epidemic. DHF cases were mostly associated with primary infection in Tainan, where no large-scale epidemic of dengue had been reported from 1944 to 1997.

Our observation of DHF in adult case-patients was different from that in dengue hyperendemic Asian countries where 80% of DHF case-patients are children (8). Dengue virus type 3 was the only serotype isolated during the epidemic in Tainan in 1998. However, this situation was similar to that in Tonga in 1974, where a dengue virus was also newly introduced (9), and to recent epidemics in south and central America (10). More adults may have been affected because fewer dengue epidemics, and therefore fewer exposures to dengue viruses during childhood, had occurred; subsequently, the immune status in adults had changed. Presumably, previous observations of severe dengue in children in Southeast Asia were the result of immunity to infection in the older population, rather than a particular susceptibility to DHF among children. Our results were not influenced by the age structure of DF and DHF case-patients in cases of indeterminate infection or in cases of sub-clinical infection in children (0.4%, unpub. data). The dengue virus with epidemic potential replicated to higher viremia titer and was associated with disease severity without consideration of immune status (11). The investigation on molecular evolution of DEN-3 virus during the 1998 epidemic in Taiwan, currently in progress, will elucidate the possible role of virus variation in the pathogenesis of DHF.

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Rift Valley Fever Encephalitis

To the Editor: Rift Valley fever (RVF) is an undifferentiated febrile illness caused by Rift Valley fever virus (RVFV). Several human outbreaks occurred in Africa, resulting in tens of thousands of infections (1,2). During fall 2000, an outbreak of RVF in the Arabian Peninsula (the first recorded outside of Africa) resulted in many human and animal fatalities (3,4). Severe and frequently fatal encephalitis thought to be directly related to viral invasion of the central nervous system develops in <1% of patients (5). Encephalitis complicating RVF is poorly described in the literature, which offers no detailed description of the clinical findings, results of cerebrospinal fluid (CSF) studies, or imaging. We describe a case of RVF encephalitis associated with retinitis, including CSF findings, viral culture results, and neuroradiology findings

An 18-year-old woman from Jazan (southwest of Saudi Arabia) had a 3-day history of confusion, fever, and blurred vision at the time an RVF outbreak was peaking in Jazan. Philadelphia-chromosome–positive chronic myeloid leukemia (CML) had been diagnosed in her several months before (leukocyte count 108 x 10^9/L). She responded well to hydroxyurea, and she had been in stable-phase CML for a few months. At the time of her visit, her temperature was 39.2°C, blood pressure 110/70 mm Hg, pulse 120 beats/min, respiratory rate 22/min. She had no lymphadenopathy, pallor, or jaundice. Results of her head, neck, and throat examinations were normal. Her chest was clear, and her abdomen was soft and nontender. Her spleen was 3 cm below the left costal margin. She was conscious and oriented. No meningeal signs could be elicited. Pupils were equally reactive to light with normal extraocular movements. Extremities had normal tone, power, sensation, and reflexes. Plantar reflex was flexor bilaterally. She had ataxic gait and bilateral retinal hemorrhages. She was unable to count fingers. Hemoglobin was 100 g/L, leukocyte count 5.1 x 10^9/L, and platelets 373 x 10^9/L. Renal and liver function tests were normal. Contrast-enhanced computed tomography (CT) scan of the brain was normal. Urine analysis and malaria smear were negative. CSF was clear. CSF glucose was 3.9 mmol/L (serum 5.8), protein 455 mg/L. CSF leukocyte count was 323 x 10^6/L, 58% lymphocytes, and 38% polynuclearleukocytes. Tests for hepatitis B surface antigen, antibodies to hepatitis C virus, HIV, cytomegalovirus antigenemia, rheumatoid factor, and antinuclear antibodies were negative. Cultures from blood, CSF, and urine were negative for bacteria. CSF viral culture was negative. Polymerase chain reaction for herpes simplex virus and enterovirus from CSF was negative. Tests for serum anti-RVF virus immunoglobulin M were positive. No other tests for RVFV were performed. Bone marrow on admission day was consistent with CML in remission. Prednisone was started on admission for 7 days.

On hospital day 5, the patient was noted to be agitated, confused, and unresponsive to commands. She was transferred to the intensive care unit after her level of consciousness decreased; she was moving all four limbs but did not respond to verbal commands or painful stimuli. Pupils were 5 mm equal bilaterally with a sluggish reaction to light. Corneal reflexes were reactive bilaterally. Gag reflex was present, and tone was increased in all four limbs with brisk reflexes and extensor planter responses. The next day, her condition deteriorated, and she became unresponsive to painful stimuli. Repeated CT scan of the brain showed no pathologic changes. Electroencephalogram showed generalized continuous rhythmic sharp and spike wave activity consistent with nonconvulsive status epilepticus. Magnetic resonance imaging (MRI) of the brain showed bilateral frontoparietal high signal intensity on T2-weighted images and evidence of subtle right posterior thalamic hyperintensity with no corresponding abnormalities in T1-weighted images. The axial diffusion MRI images were more elaborative, showing multiple bilateral asymmetrical cortical hyperintense areas consistent with an ischemic or inflammatory process. Phenytoin was started. The next day, the patient was able to open her eyes and responded to painful stimuli, corneal reflexes were present

bilaterally, oculocephalic reflex was present, and planters were flexors bilaterally. Repeat CSF studies on day 30 showed glucose of 3.8 mmol/L, protein 431 mg/L, leukocyte count of 12 x 10^6/L, and 68% polynuclear-leukocytes. Repeat CT scan of the brain showed new bilateral temporo-occipital hypodensity more on the left side and a probable right middle cerebellar and left thalamic internal capsule infarct. There was no intracranial hemorrhage or hydrocephalus. She was discharged home awake, blind, quadreparetic, and incontinent, on anticonvulsants. After 1 year, her neurologic condition had not changed.

Encephalitis and retinitis are severe complications of RVF, developing 1 or 2 weeks into the course of diseases. By that time, RVFV antigen assay is negative. Our patient met the definition of an RVF case during the outbreak (3). In one outbreak in Mauritania, 4.9% of observed infections had encephalitis (6), although the true frequency of encephalitis in RVF may be overestimated because infection can go unrecognized. The literature contains limited clinical description of this syndrome (6–8). Detailed neuroimaging findings, including MRI and flow studies, have not been previously reported. These findings, along with the patient's clinical signs and symptoms, suggest cerebral vasculitis; however, no angiogram was performed, and markers of vasculitis were negative. A more likely cause would be direct viral parenchymal invasion.

The pathogenesis of RVF encephalitis in humans is not clear. Animal studies indicate that active viral replication and necrotizing encephalitis with diffuse perivascular infiltrates of lymphocytes and macrophages occur in cerebral parenchyma (9,10). Postmortem histopathologic examination of brains of fatally infected rhesus monkeys have shown a mild, nonsuppurative, multifocal, perivascular encephalitis in the cerebral cortex, primarily of lymphoplasmacytic cells and nodular aggregates of neutrophils in association with mild necrotic changes of neurons (11). In one patient who died of meningoencephalitis in South Africa, brain pathologic findings showed perivascular cuffing and round-cell infiltration (2). In humans, we are not aware of positive RVFV cultures from CSF, blood, or brain during encephalitis. We have no reason to suspect CML to influence disease manifestations in this patient. CML was in stable phase for several months and should not have affected immune response of the patient towards RVFV.

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Lyme Borreliosis–Biology, Epidemiology and Control

J.S. Gray, O. Kahl, R.S. Lane, and G. Stanek, authors

CABI Publishing, Wallingford, Oxfordshire, UK
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347 pages, including index

Lyme borreliosis is the most common arthropod-borne disease in the United States and Europe, and cases are reported from nearly all regions of the Northern Hemisphere. As such, it is a particularly useful example of a zoonotic disease that should be considered not only from the perspective of the human patient but also from that of the vectors and reservoir hosts. This approach is the one adopted by the editors of Lyme Borreliosis—Biology, Epidemiology and Control. The first five chapters constitute a general review covering characteristics of the disease, molecular biology of the etiologic agent *Borrelia burgdorferi* sensu lato, and vector and host biology. Some of these chapters are not reviews in the truest sense. For example, chapter 2 is a primer on ecologic research; it provides important definitions and categorization of hosts and vectors. Likewise, a substantial portion of the chapter on *B. burgdorferi* in the vertebrate host is devoted to the role of complement in host specificity. Still, these chapters provide a good general description of the topics covered.

Chapters 6–9 provide more specific detail on the unique aspects of ecology in Europe, Russia, Japan, and North America, respectively. Although these chapters do not follow the same format, all cover essentially identical topics, including description of the diversity of spirochete species, vectors, hosts, landscape ecology, and risk factors. The chapter on the ecology of *B. burgdorferi* in Russia is particularly worthwhile because many of the studies described have only been published to date in Russian-language journals. This information is now available to a broader audience.

The final three chapters deal with epidemiology, prevention, and control. The chapter by Dennis and Hayes on epidemiology is outstanding and provides an excellent consideration of several controversial topics, including seronegative Lyme disease, persistent infection, chronic refractory Lyme disease, and problems of both underdiagnosis (in Lyme disease–endemic areas) and overdiagnosis. Hayes and Schriefer provide a comprehensive description of vaccine development. Unfortunately, the only commercially available vaccine, Lymerix, has been discontinued by the manufacturer, and little progress is reported on development of alternative vaccines. This leaves personal protective measures (e.g., avoiding tick-infested terrain, using appropriate clothing, checking for attached ticks), single-dose antimicrobial prophylaxis following selected tick bites, and tick control as the primary means of prevention. The last topic is covered in the book's final chapter.

The reader interested in details of the clinical aspects of Lyme disease will not find them here. Rather, the book focuses on vector and host wildlife ecology and epidemiology, as the title implies. There is an obvious effort to cover Lyme borreliosis from a global perspective rather than from a distinctly American one. This approach is one of the book's strengths, although it does lead to some bias in the selected references in certain chapters. A comprehensive index is provided, and topics covered in multiple chapters are cross-referenced well, providing an integrated feel. The book will be quite useful for ecologists, epidemiologists, and public health workers, especially those interested in vector-borne infectious diseases. The book will be less informative for those interested in detailed descriptions of the molecular pathogenesis and clinical aspects of Lyme borreliosis.

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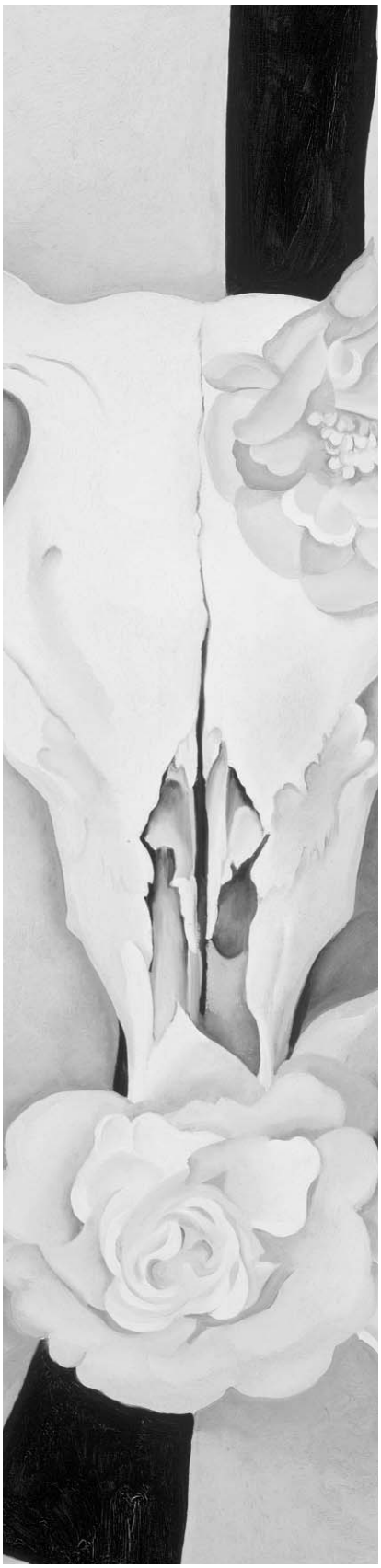

Georgia O'Keeffe (1887–1986). Cow's Skull with Calico Roses (1932).
Oil on canvas (91 cm x 61 cm). The Art Institute of Chicago

"To me they are as beautiful as anything I know," Georgia O'Keeffe said of the sun-bleached bones and skulls she found in the desert. "To me they are strangely more living than the animals walking around…. The bones seem to cut sharply to the center of something that is keenly alive on the desert even tho' it is vast and empty and untouchable—and knows no kindness with all its beauty" (1). The ragged mountain terrain with its fossilized formations, saturated color, and naked wilderness held inexhaustible fascination for O'Keeffe and was a source of inspiration for most of her artistic career.

Born in Sun Prairie, Wisconsin, the second of seven children, Georgia O'Keeffe was a pioneering and charismatic woman. Training to be an art teacher, she attended the Art Institute of Chicago, Art Students League in New York, University of Virginia in Charlottesville, and Columbia University's Teachers College. During her teaching years in Texas and later as an artist in New York City, O'Keeffe showed herself to be a complex and contradictory person with exceptional observation skills. She could fathom and depict in her work the immensity of creation in a homespun style reminiscent of what Willa Cather once called "that irregular and intimate quality of things made entirely by the human hand" (2).

Over the years, O'Keeffe became an "antiauthoritarian revolutionary," the notoriety of her lifestyle sometimes overcoming the originality of her work. She shunned European traditions and influence and resisted all manner of paternalism. Like Piet Mondrian and Kasimir Malevich, she never signed her paintings, and like Jackson Pollock, she found Native American art as inspiring as Renaissance art (3). Eventually, she abandoned the New York City art scene that founded her reputation and moved west to New Mexico for a more authentic artistic experience. There, in a landscape unencumbered by undue neighborliness and excessive vegetation, she created work that was timeless, universal, and impersonal.

From the grandeur and vastness of the western landscape, O'Keeffe extracted a compressed, concise, and reductive style. Breaking away from the constraints of scale, she painted telescopic images that favored the distant and the immediate. She made the small seem large and the large small as she focused on a single isolated object: a mountain, a stone, a flower, a bone. Educated in oriental scroll painting and influenced by the work of Wassily Kandinsky, she understood that emptiness could signify fullness, and she applied that principle in panoramic landscape paintings, as well as in lone objects placed in pictorial space (3).

Like Frida Kahlo, with whom she maintained lively correspondence, O'Keeffe became intimately familiar with her subjects, wanting to merge and become one with them at the moment of creation. "I find that I have painted my life," she confided, "… things happening in my life—without knowing" (4). Close acquaintance with the subject guided the accurate presentation not only of the outward image but also of the sensation within, transforming the subjective and personal to the mystical and universal.

"I have picked flowers where I found them," O'Keeffe acknowledged, "… have picked up sea shells and rocks and pieces of wood where there were sea shells and rocks and pieces of wood that I liked…. When I found the beautiful white bones on the desert I picked them up and took them home too…. I have used these things to say what is to me the wideness and wonder of the world as I live in it" (5). Stripping things of extraneous detail, the artist reached

for their essential geometry and substance and created images that were at once realistic and abstract.

The desert, the prairie, wide open spaces, O'Keeffe's chosen world, which inspired Cow's Skull with Calico Roses (on this month's cover of Emerging Infectious Diseases), contained all the elements essential to her art: eternal beauty, spirituality, and a timeless connection with the past. The fragile bovine skull, a ghostly remnant, hangs stark against the funereal strip in the center of the canvas. Exquisitely fine, it shares the lyricism of the floral accents and surrounding fabric folds. Its seemingly vacant visage sends an unmistakably symbolic message of death and rejuvenation.

The mythical world of the American West has had enduring allure, and not only for its artistic potential. Its vast expanses of apparently arid land, often hermetic on the surface but teaming with life, have long fascinated the naturalist, for their dust contains eons of artifacts and clues to many of humanity's puzzles.

Throughout most of western North America, from Canada to Mexico, infectious diseases peculiar to the region have been part of the landscape. With coccidioidomycosis, hantavirus pulmonary syndrome, and plague, among other vector-borne infections, the desert takes its toll. More recently, the region's famed underground "communities" of myriad prairie dogs have surfaced in the news. Exposed to a viral zoonotic agent imported from across the seas and transported around the United States, prairie dogs from O'Keeffe's adopted Southwest brought new notoriety to her native Wisconsin—site of the first outbreak of monkeypox outside the rain forests of central and western Africa.

Prairie dogs, the most social members of the squirrel family, have made their settlements from Montana to Texas and in higher elevations of the Mojave, Great Basin, and Chihuahuan deserts, posing little risk to humans as long as nature and its endemic zoonoses were in balance. Yet, import of exotic rodent species and relocation of indigenous wildlife to other areas as pets have compromised the integrity of natural cycles, proving perilous to both animal and human communities and raising the specter of interspecies transmission of infectious agents, among them those that cause tularemia and monkeypox.

Polyxeni Potter

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Ixodid and Argasid Tick Species and West Nile Virus

Pneumocystis jiroveci Dihydropteroate Synthase Genotypes in Immunocompetent Infants and Immunosuppressed Adults, Amiens, France

Epidemiologic Determinants for Modeling Pneumonic Plague Outbreaks,

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

EMERGING INFECTIOUS DISEASES

www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

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

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

Why an "Emerging" Infectious Diseases journal?

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

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

What are the goals of Emerging Infectious Diseases?

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

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

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

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

EMERGING INFECTIOUS DISEASES

EID Online www.cdc.gov/eid

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.10, No.2, February 2004

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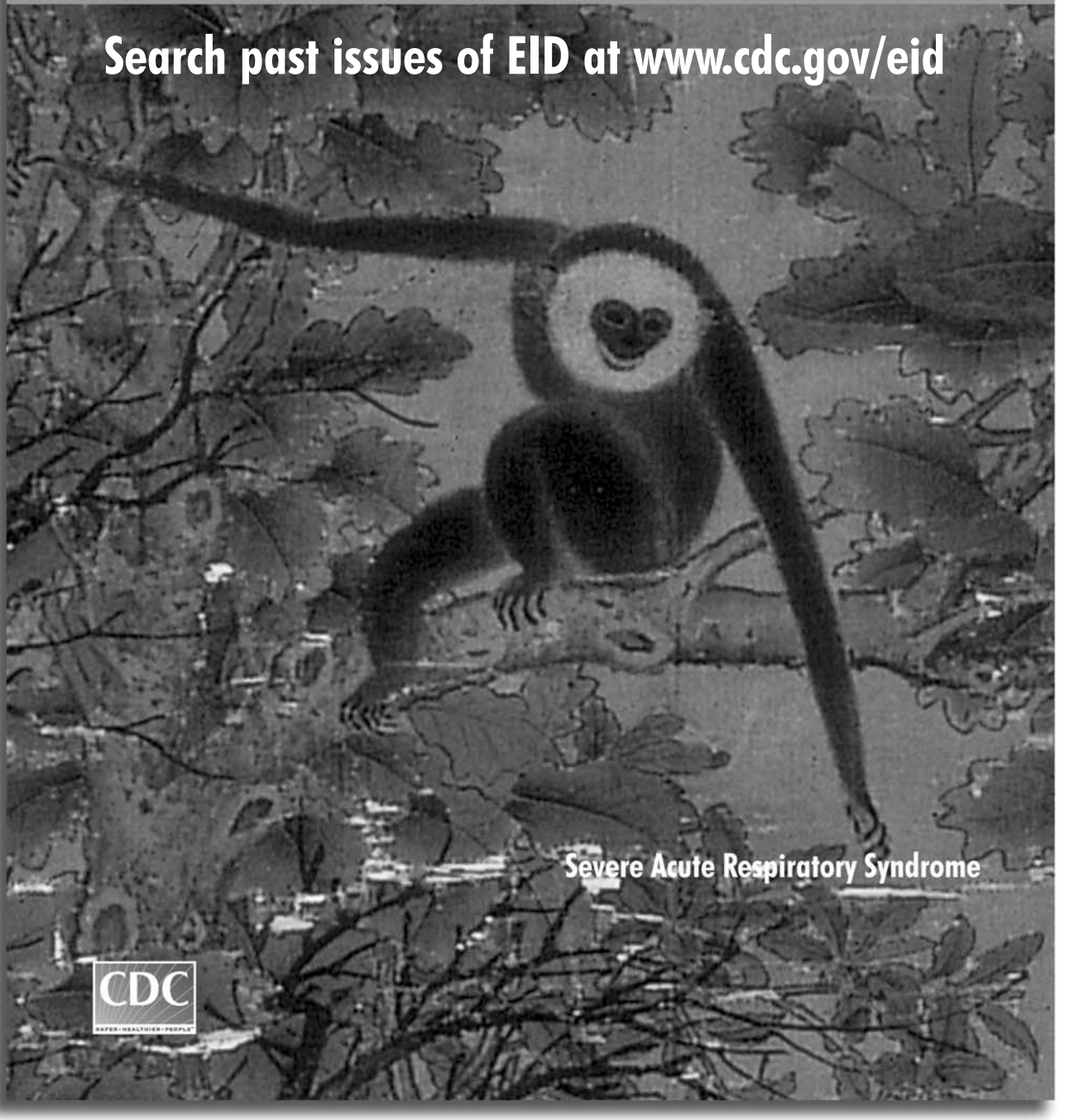

Severe Acute Respiratory Syndrome

CDC SAFER·HEALTHIER·PEOPLE

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.