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Bioterrorism-related anthrax

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An Ounce of Prevention Is a Ton of Work: Mass Antibiotic Prophylaxis for Anthrax, New York City, 2001

Susan Blank,*† Linda C. Moskin,† and Jane R. Zucker*†[1]

Protocols for mass antibiotic prophylaxis against anthrax were under development in New York City beginning in early 1999. This groundwork allowed the city's Department of Health to rapidly respond in 2001 to six situations in which cases were identified or anthrax spores were found. The key aspects of planning and lessons learned from each of these mass prophylaxis operations are reviewed. Antibiotic distribution was facilitated by limiting medical histories to issues relevant to prescribing prophylactic antibiotic therapy, formatting medical records to facilitate rapid decision making, and separating each component activity into discrete work stations. Successful implementation of mass prophylaxis operations was characterized by clarity of mission and eligibility criteria, well-defined lines of authority and responsibilities, effective communication, collaboration among city agencies (including law enforcement), and coordination of staffing and supplies. This model can be adapted for future planning needs including possible attacks with other bioterrorism agents, such as smallpox.

As part of national bioterrorism preparedness efforts, New York City began actively developing protocols for the distribution of mass antibiotic prophylaxis against anthrax in early 1999. These efforts were led by the Mayor's Office of Emergency Management, in close collaboration with the New York City Department of Health (DOH). The goal of the plan was to have the ability to provide mass antibiotic prophylaxis to 8 million New Yorkers over a 48-hour period—in the worst-case scenario of a large-scale bioterrorism attack—without impinging upon the capacity of local medical facilities to respond to the needs of persons affected by the biological agent. Here, we highlight aspects of New York City's emergency planning, the circumstances of the six actual implementations in the city in 2001, and the lessons we learned.

Planning

New York City's government agencies, including DOH, are part of an incident command structure that reports to the Mayor's Office of Emergency Management during public emergencies (1). In 1999, this department established an internal incident command structure, composed of the following: clinical response, sheltering, surveillance, environmental health, laboratory, communications, management information systems, and physical plant operations components. "Round-the-clock" coverage was adopted at all agency levels. These teams are operated by persons from a variety of the city's DOH programs.

Chronology of Events

On October 9, 2001, New York City's DOH was notified of a possible case of cutaneous anthrax in a female staff member of a nightly news team at a large media company (Table 1). On October 10, the department's incident command system was put into effect, and team leaders were informed of the situation. From then until October 12, 2001, when the diagnosis was confirmed, DOH finalized an antibiotic distribution plan, including development of a medical charting system, standing orders for dispensing antibiotics, training curricula for staff, and reproduction of antibiotic fact sheets (in English and Spanish). Clinical materials were reviewed by the department's general counsel, and scripts were developed for information hotlines. DOH staff were identified and reassigned to this effort.

On October 12, 2001, the department began collecting nasal swabs and distributing prophylactic antibiotics to persons working at the media company who might have been exposed to a letter implicated in the index case. Included in this effort were those working on the same floor as the index patient. Initially, the exposure source was believed to be a letter postmarked September 25, 2001, potentially exposing an estimated 200 persons. This letter was tested for *Bacillus anthracis* multiple times, however, and all tests were negative (2).

Within hours of the Mayor's public announcement of this case, DOH and the Office of Emergency Management

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[1]All three authors contributed to the concept and design of this paper. Susan Blank wrote the first draft. Major editings and additional material were contributed by Linda Moskin and Jane Zucker.

Table 1. Chronological summary of six anthrax events requiring PODs

Event	Location	No. of eligible persons registered	Total hours of operation	Briefing format for eligible persons oral/written	Antibiotics	Nasal swabs taken
1	Media 1	1,322	42	No/yes	Yes	Yes
2	Media 2	763	36	No/yes	No	Yes
3	Media 3	175	25	Yes/yes	No	Yes
4	Media 4	354	14	No/yes	No	Yes
5	USPS	7,081	67	Yes/yes	Yes	No
6	Hospital	1,923	28	No/yes	Yes	No

[a]POD, point of distribution (for antibiotics); USPS, U. S. Postal Service.

established an antibiotic distribution site (referred to as a point of distribution [POD]), at the main building that housed the media company. The space provided for prophylaxis was in the same building complex that housed the letter but did not share the ventilation systems that served the areas in the letter's path. The layout of the space provided for the POD and its operations could not accommodate large groups of people seeking antibiotics. Moreover, the letter was a matter of a criminal and epidemiologic investigation, so law enforcement agencies needed to conduct their own interviews on site. Thus we coordinated with law enforcement personnel to minimize disruption of client flow and ensure that client medical confidentiality would not be compromised. The epidemiologic aspects of the investigation were initially incorporated into the medical record used.

Soon after distribution of antibiotics was begun, the source of anthrax was confirmed to be a letter postmarked September 18, 2001. Consequently, the time interval during which exposure may have occurred was reevaluated and the number of people possibly exposed substantially expanded.

Between Friday, October 12, and Tuesday, October 16, after approximately 42 hours of operation and an average of 55 staff persons per shift, 1,322 persons were briefed, completed epidemiologic and law enforcement interviews, underwent medical assessments, had nasal swabs taken to better define exposures, and were given a 14-day supply of antibiotics within the POD space. The average throughput time (the time from a client's entry into the POD space to exit) was 30 minutes per client. Initially, the briefing of staff consisted of providing written materials. This system was augmented by a combination of information distributed over closed-circuit television throughout the still-operating company and by direct electronic communication from the company's senior management. Within the first day of operations, it became apparent that both potentially exposed and unexposed persons needed emotional support and further information about the event, the risk for anthrax exposure, and the dangers of antibiotic misuse. Counselors (medical and mental health) were made available immediately outside the POD, and hotline staff were given scripts to assist them in answering concerned callers. The city's DOH supplied each potentially exposed person with an initial 2-week course of antibiotics to provide time for public health officials to complete the investigation and develop specific criteria for persons needing to complete the balance of the 60-day prophylactic regimen.

Once the investigation was complete (October 20, 2001), DOH narrowed the criteria for antibiotic prophylaxis to those met by the 12 persons who directly handled the contaminated letter and recommended that all others discontinue antibiotics. This general information was communicated by the employers to all antibiotic recipients and by letters mailed from DOH to affected persons. We also directly contacted all 12 persons who needed to continue prophylaxis. Ultimately, 60-day inhalational anthrax prophylactic regimens were provided to 11 persons (6 working in the building and 5 involved in the recovery of the tainted letter) largely by means of the on-site employee health unit. One person refused prophylaxis. We later assisted the Centers for Disease Control and Prevention in evaluating adherence and adverse drug effects among those receiving 60-day regimens.

Four cutaneous anthrax cases were subsequently identified in New York City; these cases occurred at three other media outlets (one case each at two locations and two cases at the third) (3). All of these cases were believed to be associated with contaminated mail. No inhalational anthrax cases were associated with the media outlets. These three PODs served persons potentially in direct contact with the suspect letters. POD activities, however, were restricted to registration, provision of printed information, epidemiologic interviews, and obtaining of a very limited number of nasal swabs within the POD space. Subsequently, the decision to provide antibiotics was based on confirmed exposure, as determined by the epidemiologic investigation. Antibiotics were dispensed on an individual basis, as was monitoring for adherence and adverse events. Epidemiologic and law enforcement interviews and large informational sessions for all staff were held separately, in separate locations, and at different times from those for the POD. Counseling was available immediately after the information sessions or thereafter through the DOH anthrax hotline.

The fifth POD was conducted in New York City by the U.S. Public Health Service (USPHS). This site's purpose was to provide an initial 10-day course of antibiotics to prevent inhalational anthrax in ~7,000 postal employees who worked at facilities that processed the anthrax-containing letters sent to the above referenced media outlets (events 1–4). Although no anthrax cases had been reported among the city's postal workers, inhalational anthrax cases had occurred in postal workers in New Jersey and in the Washington, D.C., area (4). Anthrax spores were subsequently found in one of New York City's postal facilities. Both labor and management at affected facilities requested prophylaxis for inhalational anthrax. As these were federal facilities and federal employees, prophylaxis efforts remained in the jurisdiction of the federal government. The POD was conducted by USPHS in the basement of a New York City mail-processing center (5). U.S. Postal Service management was instrumental in securing space and identifying and scheduling staff. USPHS determined the initial operational layout, medical charting, and staff needed for this effort on the basis of its prior experiences in the Washington, D.C., area postal facilities. Additionally, written information was deemed insufficient for this setting. The increased throughput time reflects the inclusion of extensive live briefings accommodated within this POD space.

Liaisons from DOH's clinical response team were assigned to this effort as consultants. DOH's role was limited to increasing the efficiency of POD operations. We assisted USPHS effort by providing detailed clinician training materials, medication fact sheets, and on-site flow analyses with recommendations to improve client throughput on the basis of local POD experiences. Collaborative efforts also included the timely sharing of information with DOH for response to public inquiry, DOH assistance in establishing local medical and mental health referral patterns, and follow-up of these referrals. USPHS, in turn, accommodated visits to the operation by members of the New York City Office of Emergency Management and DOH staff for educational purposes.

Between Wednesday, October 24, and Saturday, October 27, in approximately 67 hours of operations with 65–70 staff persons per shift, this fifth POD provided 7,081 persons with a 10-day supply of antibiotics. The POD provided registration; completion of a medical screening form; detailed live briefings on risk for exposure, signs and symptoms of anthrax, and side effects of the recommended antibiotics; medical screenings; and antibiotic distribution. The average throughput time for these activities was 33 minutes per client. In addition to the medical personnel who were on site to evaluate symptoms and adjust antibiotic regimens, staff were available for mental health and other counseling issues. Epidemiologic and law enforcement interviews were conducted separately; no nasal swabs were collected because >30 days had elapsed since the suspect letters were processed. The federal agencies directly managing prophylactic efforts subsequently offered additional prophylactic antibiotics with or without the anthrax vaccine to those persons thought to have been most highly exposed to aerosolized *B. anthracis*.

On October 28, 2001, DOH was notified of a case of inhalational anthrax in a 64-year-old woman working in a hospital stockroom. The patient had no discernable association with the media companies or the postal service, although a section of the stockroom where she worked was adjacent to the hospital mailroom (6,7). While environmental samples were being collected, DOH immediately set up a POD (event 6) for hospital staff, patients, and visitors who had spent >1 hour in the hospital since October 11 and thus might have a risk for exposure to aerosolized *B. anthracis.* During the environmental investigation, the hospital was closed.

Between Monday, October 29, and Friday, November 2, over the course of 28 hours and with a staff of 53 persons per shift, 1,923 persons received prophylactic antibiotics. Epidemiologic and law enforcement interviews were targeted to include only hospital staff. Nasal swab specimens were collected from 28 persons who worked in and around the mailroom. The average POD throughput time was 6½ minutes per person. POD activities involved registration, triage, medical evaluation, dispensing antibiotics, counseling, and overall management. No informational sessions were conducted; however, written information (including DOH hotline telephone numbers) was distributed, and counseling staff were available. Nasal swabs were not routinely collected. This POD, which was situated in a hospital and focused on hospital personnel, was facilitated by close collaboration with the hospital administration, which helped coordinate prophylaxis efforts and mobilize hospital staff to assist in POD operations.

Antibiotic distribution was discontinued on November 2, when all environmental samples from the hospital tested negative for *B. anthracis.* By mail, DOH informed all persons provided with antibiotic prophylaxis to discontinue their regimens.

Discussion

Planning versus Reality

Prior emergency planning addressed large-scale events affecting 8 million New York City inhabitants; under those circumstances, ordinary medicolegal considerations would not apply (e.g., no provider-patient relationship invoked; no need for medical charting, nonprofessionals used for staffing). Our PODs were initiated before the extent of

exposure was known and were later limited to those persons most likely to have been exposed. Clearly, the intentional release of anthrax affected far fewer than the projected worst-case scenario.

Consequently, our PODs were more “classically” modeled, i.e., they included a large staff of licensed medical professionals who obtained consents, took medical histories, collected specimens, and dispensed antibiotics. A full medical charting system was available, as were mental health and medical counseling services, at each site. These services were augmented by toll-free hotlines.

Client Screening, Functional Units, and Flow Patterns

Clients were persons meeting eligibility criteria for receiving antibiotics and thus granted access to the POD. Ineligibles, or the “worried well,” were persons who did not meet the eligibility criteria to enter the POD or receive antibiotics within the POD; they were offered informational materials, the opportunity to speak with counselors, and access to the DOH public hotline.

The POD proper is defined as the space where patients are registered, triaged, have swab samples taken (as necessary), evaluated medically (as necessary), and provided with antibiotics. Other POD-related activities (which may or may not be part of the layout of the actual POD space) include assessing the eligibility of persons who present themselves, reassuring the worried well, briefing clients about anthrax and POD operations, collecting information for investigative purposes, transferring persons to a medical facility (when needed), counseling, managing client flow, and maintaining security.

Immediately outside the entrance to the POD, we placed a screening station, where POD staff verified eligibility and gave eligible persons writing tools, an information sheet, the epidemiologic interview form, the law enforcement interview form, and a medical record form to complete. Articulating clear eligibility criteria and obtaining verifiable lists of names of persons expected at the POD (including relevant contractors such as housekeeping and house security) helped maintain order at the front door. As these events occurred in occupational settings, management was critical in communicating public health messages to staff, identifying and scheduling staff access, and setting clinic hours to maximize the flow of the prophylactic effort; strong management resulted in organized PODs and responses.

Because bioterrorism is a criminal act, law enforcement agencies had a separate and independent purview for investigation. Performing investigative interviews first and separately from the POD proper alleviated concerns about maintaining client medical confidentiality and facilitated client flow, although this was dictated largely by the layout and physical capacity of the space allotted. Furthermore, investigative interviews involved more well-defined and smaller subsets of persons with each subsequent POD.

The client registration process also evolved with each POD. Initially, identifying data were handwritten in a logbook. This system was supplanted by the use of a single spreadsheet on a laptop, and finally, by the second day of the first POD, by several laptops with data-entry screens and wireless connections to an on-site server. These adaptations permitted rapid tracking of clients served and facilitated subsequent correspondence through the production of mailing labels. This system was upgraded and used at subsequent DOH PODs. As we quickly adopted a computerized registration process, management information system staff provided on-site technical support.

After registration, clients moved into the triage area, where an assessment was made about whether they could proceed directly to the dispensing station or needed to be medically evaluated before a final determination on prophylactic antibiotics could be made. All clients not eligible for immediate receipt of prophylactic antibiotics were triaged to the medical evaluation area. There, staff (physicians, nurses, and physician assistants) determined the appropriate prophylactic medication choice or need for further evaluation and transfer to a healthcare facility. Because a limited number of circumstances require alteration of the prophylactic regimen or of a client’s original medication regimen (~10% of all clients), we created a clinical algorithm and preprinted instruction sheets for those situations.

Antibiotics were distributed at the dispensing station, as were fact sheets explaining antibiotic use. This station was staffed by nurses, physicians, or pharmacists, as resources permitted. Having at least one pharmacist present proved useful.

Because some clients were overwhelmed by the situation and had residual questions, mental health, medical advisors, and public health educators were available at the POD entrance and near the POD exit for consultation. Also, by referring persons to the hotline and website, we limited the need for on-site counselors.

Security is an essential feature of POD operations. The New York City Police Department provided this service. Officers maintained order at the entrance and exit, so that the POD was not overwhelmed with anxious and angry persons (either those at risk or the worried well), and guarded pharmaceutical supplies.

Persons devoted solely to ensuring the smooth flow of clients into the POD, from one area (or station) to the next, and out of the POD also were essential. These flow managers, or “traffic” personnel helped minimize client-waiting times and staff idle time and improved throughput times. An area for clerical staff to manage medical charting within the POD also was necessary.

Tailored POD Elements

Each POD was conducted differently, combining a standardized response for anthrax prophylaxis with the unique needs of each setting. Services provided within the POD space varied. The space allotted, the POD staffing available, preexisting circumstances (e.g., organizational structures, historic relations between labor and management, client characteristics), and ongoing field assessments determined the array of services offered. Similarly, POD work-shifts were defined on the basis of need, resources, and input from representatives of those affected.

Staff and Space Needs

The most important element for an efficient POD process is adequate staffing to operationalize antibiotic distribution and to ensure that anticipated language needs of the clients are met. Ideally, an organizational diagram should be in place, along with a brief description of the role of each staff member and any training documents necessary. The organizational chart we found most useful is shown in Figure 1. Four critical positions are the executive liaison, physician-in-charge, supplies coordinator, and clinic manager; their primary responsibilities are outlined in Table 2. We learned that the physician-in-charge should be dedicated solely to running the POD. A second public health officer should be on-hand to convene regularly with key representatives of potentially exposed populations.

The POD site should be conveniently located for those affected but should not be located in a place that might be contaminated with *B. anthracis*. Ideally, site options should be considered well before the need for such a site arises. Selecting a space and arranging stations to promote continuous flow of clients (including the disabled and children in strollers) proved important. To distribute antibiotics to 500 to 10,000 clients over a 72-hour period, a space of at least 2,500 square feet for the POD proper was necessary. To minimize the impact of unanticipated space issues, we subsequently developed several possible floor plans, so that a quick assessment of layout could be made during subsequent site selection (Figure 2, A and B) and a predesigned floor plan could be adapted to a particular situation. Despite the urgency of the situation, allocating adequate time before opening a POD is critical to ensure that supplies have arrived and trained staff are ready to begin operations.

Client Flow through the POD

Our PODs operated most efficiently when activities were handled at discrete workstations. As we progressed though the six events, we also realized the utility of physically separating activities for which clients may need to sit from those that did not require sitting. It was most time-efficient if clients did not sit to receive clinical services, and most space-efficient if no chairs were available for clients. Paperwork and interviews were best suited to occur outside the POD proper, since clients found it easier to fill out forms and participate in interviews while sitting. Thus space allocation should also include a space, preferably outside the POD proper, for these "seated" client activities (i.e., filling out forms and conducting interviews) to ease the difficulties of moving people through the POD. Persons able to perform briefings and translators (including those skilled in sign language) should be available in this area to assist with questions.

Streamlined Medical Chart

The medical chart was revised between events. Initially, we obtained very structured medical histories and collect-

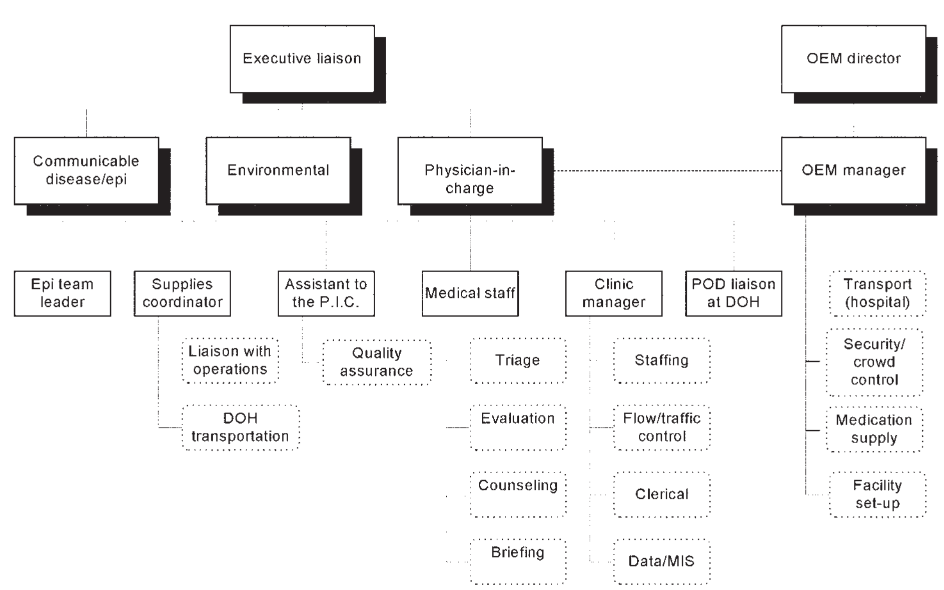


Figure 1. Point of distribution (POD) site organizational chart. OEM, Mayor's Office of Emergency Management; PIC, physician-in-charge; Epi, epidemiologic; DOH, Department of Health. Dotted boxes = areas of responsibility; dotted lines = shared communications.*For operational purposes, the epidemiologic team leader reported to the PIC.

Table 2. Job titles and primary responsibilities recommended for PODs

Job title	Primary responsibilities
Executive liaison	Reports to the incident commander
	As senior staff member in the field, coordinates both the investigation (epidemiologic and environmental) and the prophylaxis effort
	Interfaces between the public health agency and the organization representing those to receive prophylaxis
	Ensures that the physician-in-charge is informed of recent developments of the investigation, as well as other information from Department of Health command center briefings (i.e., changes in treatment recommendations, eligibility criteria, or reports of organism antibiotic susceptibilities)
Physician-in-charge	Reports directly to the executive liaison, keeping him or her appraised of progress and problems
	Is responsible for the overall POD operations, including site selection, POD set-up (including floor plan and staff training), ensuring communication among POD stations, and overseeing collection of epidemiologic and law enforcement data
	Is responsible for on-site oversight of the epidemiologic investigation, the supplies coordinator, the medical service staff (e.g., physicians, nurses, pharmacists, mental health professionals), and the clinic manager
Supplies coordinator	Ensures that all forms, supplies, and equipment are available at the POD when needed (prepared in advance, supplied to POD, and replenished as needed)[b]
	Is responsible for transportation of staff and material
Clinic manager	Oversees nonclinical operations within the POD, such as staffing, patient flow, clerical, and MIS operations, communications, medical records retention, and quality improvement activities
	Coordinates activities with the supplies coordinator

[a]POD, point of distribution (of antibiotics); MIS, management information systems.

[b]Supplies to be provided include general supplies (medical charts, epidemiologic questionnaires, preprinted training instructions for staff at various stations, literature for patients and staff, medical charts, office supplies, white coats, and other clothing with appropriate insignia for nonclinical personnel), laboratory supplies (if needed, nasal swabs, laboratory requisitions forms, specimen bags, specimen labels, water-free hand sanitizing solution, and disposable laboratory gowns, gloves, and biohazard bags), and pharmaceutical supplies (antibiotics [in adult and pediatric dosages], a copy of the Physician's Desk Reference, and medication fact sheets for each drug to be dispensed).

ed nasal swabs from all clients, creating tremendous delays. With subsequent PODs, we redesigned our medical chart to be a one-page (two-sided), self-administered questionnaire, limited to information relevant to the rapid distribution of antibiotics. The chart included personal contact information (e.g., address, telephone numbers, and identification of emergency contacts), a signed consent form for testing and treatment, brief medical history (presence or absence of current anthrax symptoms, relevant drug allergies, use of specific medications known to interact with doxycycline or ciprofloxacin, pregnancy status), as well as a place to document nasal swab collection, the dispensing and receipt (or refusal) of antibiotics, and antibiotic lot numbers. A separate medical record was created for pediatric clients and followed the same general formatting. Also, as the utility of nasal swabs became better understood, DOH progressively restricted the epidemiologic criteria for obtaining them, relieving an important system bottleneck at triage.

Short Briefings

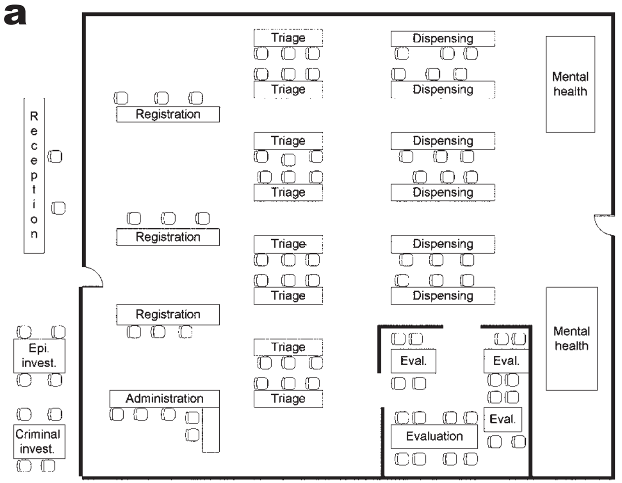
If necessary, live briefings need to be short and should include multiple briefing stations with good sound systems. Staggered briefings (i.e., 7–10 minutes in length, beginning every 5 minutes) helped distribute client flow. Including information on antibiotic dosage and side effects in these briefings was useful. Persons able to perform briefings and translators (including for sign language) should be available in this area to assist with questions. Clients may be provided with a written information sheet in lieu of a briefing, a step that improves client flow; a counselor can be available to handle further questions.

Communication

Careful attention to communication at a variety of levels is critical, including from the incident command center to the POD and from the health department to the public and to community medical providers. Also important was the flow of information from public health officials to representatives of the community receiving prophylaxis, and to the community itself. Without such attention, centrally made decisions might not be communicated to POD staff, resulting in mistaken expectations.

Cell phones and two-way radios were important means by which to communicate. Electronic mail was not available for POD staff. Materials initially developed required continuous updating of facts, whether or not new information was available (e.g., "There are no new cases of anthrax as of today."). These materials needed to be appropriate for public use. Materials were used at POD sites, for DOH hotline scripts, and on the DOH website. Information was also disseminated by means of press releases and press conferences.

The format for communicating with POD clients—including printed materials, live briefings, or both—was decided jointly by management and public health officials on the basis of resources, the extent and severity of actual cases, and knowledge level of the clientele. The medical community was kept abreast of recent developments through multiple broadcast faxes, emails, and website



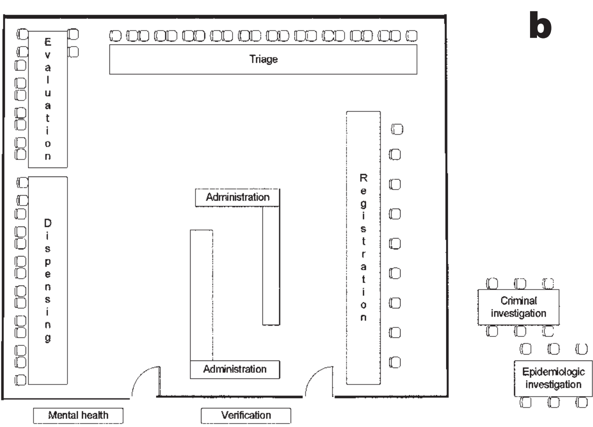


Figure 2. Two (a, b) point of distribution site floor plans. Epi, epidemiologic; invest, investigation; admin, administration; eval, evaluation; Disp., Dispensing; Reg, registration. b, floor plan of POD proper. The verification, epidemiology investigation, and criminal investigation sections are located before the POD proper. The mental health and briefing sections are also located outside the POD proper.

updates from DOH and by quickly establishing a DOH physician hotline staffed by medical professionals. In summary, DOH established three separate hotlines, one each for physicians, those clients directly affected by POD operations, and the general public.

Preplanning

Events that require a POD (i.e., intentional dissemination of virulent organisms) are stressful for all involved. In a public health emergency, little time exists between the decision to open a POD and initiation of operations. Planning can help alleviate the need to make decisions under pressure and can ensure quality of effort. The interval before opening the POD can then be used for truly last-minute preparations: mobilizing and orienting personnel, finalizing briefing sheets, and selecting a POD location and layout.

Training should also begin well before an emergency actually occurs so that staff assigned to assist with POD operations are familiar with the process, forms, and data-entry screens and have a personal emergency plan in place (e.g., child and pet care, transportation) to accommodate an altered work schedule. Having each staff person's tasks be limited enough to be "digestible" in a short orientation session at the time of POD operations is also helpful. The local health department can prepare for mass prophylaxis efforts by developing a standing set of employee rosters for round-the-clock coverage in 12-hour shifts, with approximately 50–55 persons per shift. This schedule enables antibiotic prophylaxis to be provided to up to 10,000 persons in 72 hours.

Advance Resource Building

A major difficulty in staffing a POD with health department staff, especially in small health departments, is that these staff are removed from their regular duties. One approach to minimize the effect on single programs is to compose teams from a variety of programs. Another strategy is to use staff from preexisting program groupings, with existing work relationships.

Most health departments are not poised to handle single large POD efforts (>10,000 persons) or even multiple concurrent ones for <10,000 persons. To preserve the integrity of public health functions during large or concurrent POD mobilizations, partnerships are necessary to mobilize qualified personnel from a variety of resources in and around the affected community. Thus, health departments need to have established relationships with other organizations (e.g., Visiting Nurse Service, American Red Cross) that can assist if needed. Any mobilization across agencies will be facilitated by prior communication and coordination on issues such as deputization,[2] licensure, medicolegal responsibility, and payment of wages.

Allocation of Resources

Relationships arose during the POD events that made important resources available. Our prophylaxis efforts took place in occupational settings, primarily for employees at these settings. Management and labor representatives were important assets for facilitating POD operations.

[2] Deputization formally gives a volunteer responsibilities and privileges during the temporary assignment as an agent of DOH. Responsibilities include following DOH rules on confidentiality, handling medical records, making decisions on DOH's behalf, and stewarding resources (especially medications and equipment) according to DOH protocol. DOH will in turn offer some protections (e.g., proper equipment, malpractice coverage, worker's compensation coverage).

DOH tried to limit antibiotic distribution to those who needed them; the department used the opportunity to educate the public on the hazards of inappropriate antibiotic use. Our role was to ensure access to antibiotics, educate POD clients of the need to complete the prescribed regimen once started, and ensure that the health department maintained critical public health functions. The concern with anthrax is primary, not secondary, spread, and as such, precious public health resources should not be used to ensure adherence on a case-by-case basis.

Conclusion

A successful POD is characterized by clarity (clear mission and eligibility criteria, clear lines of authority, clearly defined responsibilities, clear antibiotic recommendations); communication (between the DOH incident command on site at the POD and organizations representing those receiving prophylaxis); collaboration (with other agencies that may be called upon to assist in delivery of prophylaxis and law enforcement agencies needing to gather information about the crime scene); coordination of staffing and supplies; and prudent choice of POD site. Future planning should include scenarios that address alternative prophylactic modalities (e.g., immunization, especially for smallpox), on-site infection control needs (such as use of masks or isolation for symptomatic persons), and automated management information systems for more efficient operations.

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Inactivation of *Bacillus anthracis* Spores

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After the intentional release of *Bacillus anthracis* through the U.S. Postal Service in the fall of 2001, many environments were contaminated with *B. anthracis* spores, and frequent inquiries were made regarding the science of destroying these spores. We conducted a survey of the literature that had potential application to the inactivation of *B. anthracis* spores. This article provides a tabular summary of the results.

In October 2001, several letters containing *Bacillus anthracis* spores were sent through the U.S. Postal Service to recipients in government and private-sector buildings. Consequently, 23 human inhalational or cutaneous anthrax infections occurred. Five of the 11 inhalational anthrax infections were fatal (1,2). As a result of this intentional release of *B. anthracis*, several post offices, mailrooms in government buildings, and private office buildings were contaminated with *B. anthracis* spores. During the initial response, frequent requests were made for published materials about inactivating *Bacillus* spores. However, no adequate single source of literature on this subject was available. Because of the risk to humans, remediation of anthrax-contaminated buildings and their contents has been the focus both of scientific discussion and commercial product marketing. A number of manufacturers have developed equipment or materials that reportedly kill *B. anthracis* spores. However, these manufacturers have tested their products with laboratory tests that use *Bacillus* species other than *B. anthracis*, and the efficacy of some of these technologies relies on published literature. An obvious concern is whether postremediation levels of spores are safe; the summarized studies make no claim about whether a safe level exists and what it might be.

We provide a summary of much of the available literature on the inactivation of *Bacillus* spores that is relevant to the inactivation of *B. anthracis*. We reviewed publications from 1930 to 2002, and we have created a tabular summary of those articles. Treatments or agents commonly cited include heat, formaldehyde, hypochlorite solutions, chlorine dioxide, and radiation. Methods regarding inoculum size, concentration, and other variables are not consistent between experiments, but each experiment provides some specific information of value. Early studies that lack quantitative data are not included. A number of the cited studies address *Bacillus* species other than *B. anthracis*. We include these for information, with the caveat that surrogates do not always predict the behavior of the target species. Furthermore, the results from laboratory experiments do not specifically address questions regarding the best methods for inactivating spores on different materials such as mail, carpet, other porous objects, food, or water. Transfer of these sporicidal methods from the laboratory to a building has not yet been tested; however, the known laboratory results are a logical place to start when considering the decontamination of a building.

Decontamination is defined as the irreversible inactivation of infectious agents so that an area is rendered safe. However, decontamination may not eliminate bacterial spores. Sterilization is the complete destruction or elimination of microbial viability, including spores (3).

The experiments described provide a logical starting point for future experiments and decontamination strategies in the event of anthrax bioterrorism. Our intent is not to provide a comparative evaluation or recommendations for decontamination but rather to summarize the quantitative published results and provide a useful reference.

Review

Variations in time, temperature, concentration, pH, and relative humidity may affect the sporicidal activity of various agents. Accordingly, and especially for real-world situations, attention must be paid simultaneously to more than one controllable or uncontrollable factor. In Tables 1 and 2 and in the discussion, we address some of the key ancillary factors.

Boiling water for >10 minutes, for example, can reduce *B. anthracis* spore counts by at least 10^6 (Table 1). Variations in time and temperature conditions required to reduce spore counts listed in Table 1 can be attributed to differences in experimental conditions, strains of *B. anthracis* tested, or inoculum size.

The U.S. Environmental Protection Agency indicates that use of sodium hypochlorite as a sporicide is applicable under an emergency exemption (Section 18: Crisis Exemption; Federal Insecticide, Fungicide, and

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Table 1. Heat inactivation of *Bacillus anthracis* spores

Temperature	Time	Inoculum size	Inactivation effect	Ref.
Boiling				
100°C	10 min	3×10^6	Sample sterilized	4,5
	5 min	7.5×10^8	Sample sterilized	
Moist heat				
90°C	20 min	1.2×10^6	Sample sterilized	4,5
90°C to 91°C	60 min	3×10^8	Spores detected	
100°C	10 min	1.2×10^6	Sample sterilized	5,6
100°C to 101°C	17 min	1×10^5	Sample sterilized	
105°C	10 min	3×10^6	Sample sterilized	5
120°C	15 min	2.4×10^8	Sample sterilized	4
Dry heat				
140°C	>90 min	6×10^3 to 1.2×10^4	Sample sterilized	7
150°C	10 min	6×10^3 to 1.2×10^4	Sample sterilized	
160°C	10 min	6×10^3 to 1.2×10^4	Sample sterilized	
180°C	2 min	6×10^3 to 1.2×10^4	Sample sterilized	
190°C	1 min	6×10^3 to 1.2×10^4	Sample sterilized	
200°C	30 sec	6×10^3 to 1.2×10^4	Sample sterilized	

[a]Spores in liquid suspension exposed to flowing steam at 100°C.

Rodenticide Act); as such, sodium hypochlorite may be used under the conditions specified (32). Given these conditions, the sporicidal effectiveness of hypochlorite solutions depends on the concentration of free available chlorine and pH. Common household bleach (sodium hypochlorite) has a pH of 12 to prolong its shelf life. To achieve effective sporicidal activity, bleach must be diluted with water to increase the free available chlorine and acetic acid to change the pH of the solution to 7 (11). Organic matter may decrease the sporicidal efficiency of sodium hypochlorite (33).

Concentration, humidity, temperature, and carrier material affect gaseous sterilization of spores. Ethylene oxide penetrates into porous material (absorbed strongly by rubber and many plastics); thus vapors are not readily eliminated by brief aeration. Ethylene oxide is also flammable (34). Residual spores were not completely killed after a 30-minute exposure to chlorine dioxide at a relative humidity of 20% to 40%, whereas all spores were killed after a 15-minute exposure to chlorine dioxide with the addition of prehumidification at a relative humidity of 70% to 75% (21). Peracetic acid vapor does not penetrate well into porous surfaces and is flammable. The amount of contamination, level of cleanliness of surfaces, and relative humidity will contribute to peracetic acid vapor's effectiveness as a sporicide (24). Organic matter may absorb and chemically react with propylene oxide, reducing its effectiveness. Organic matter may also provide physical protection from the oxide (25). The sporicidal property of ozone is affected by relative humidity: as relative humidity decreases, the time required for killing organisms increases (27).

Discussion

Decontamination of buildings from intentional release of *B. anthracis* is a new problem, and no accumulated scientific knowledge exists on the subject. Two areas of prior scientific research may be relevant: food processing and laboratory decontamination. With modification based on further study, the technologies used in laboratories and food processing plants may be applied to buildings.

Direct information on killing *B. anthracis* spores in foods by cooking is scarce, and the complexity of food matrices precludes easy extrapolation of the laboratory data into nonfood matrices. However, information on inactivating spores of bacterial species more resistant to environmental conditions than *B. anthracis* can provide guidance. The spores of *Clostridium botulinum* are more resistant to heat inactivation than are *B. anthracis* spores (4). The commercial retort process of canning achieves a 12-log reduction of *C. botulinum* spores, and by extension, should achieve a similar killing rate for *B. anthracis* spores. Further research in this area is needed.

Historically, formaldehyde solution or gas has been used both as a disinfectant and chemical sterilant. Formaldehyde was used to disinfect as early as the late 1880s and is still used to reprocess hemodialyzers for reuse on the same patient and to decontaminate biologic safety cabinets and laboratories (35–37). Formaldehyde gas has been used for fumigation in the poultry industry and for disinfection of biologic safety cabinets and laboratories (38,39). Data from controlled experiments with *B. globigii* NCTC 10073 spores have demonstrated the effect of humidity on formaldehyde concentration (mg/m^3) to obtain a >8-log reduction in viable spores (15).

Fumigation with formaldehyde vapor (18 mg/L–21 mg/L) has also been used to treat a textile mill contami-

Table 2. Efficiency of chemicals, gases, and radiation on the inactivation of *Bacillus* spores

Method	Concentration	Inoculum size	Time	Efficiency	Ref
Chemical sterilization					
Calcium hypochlorite	20 ppm available; Cl_2, pH 8.0, 20ºC	3 x 10^5–4 x 10^5 spores of *Bacillus subtilis* in 5.0 mL sterile distilled H_2O	4.8 min	99% killed	8
	25 ppm available; Cl_2, pH 6.0, 20ºC	2 x 10^7 spores/mL of *B. metiens* in 10 mL of sterile distilled H_2O	2.5 min	0.061 (log of average % survivors) 99% killed	9
Free available chlorine	2.4–2.3 mg/L available; CL_2, pH 7.2, 22ºC	1.1 x 10^5 spore suspension of *B. anthracis*	1 h	>99.99% killed (1 spore/mL survived)	10
Sodium hypochlorite (NaOCl)	0.05%, pH 7.0, 20°C	Spore suspension of *B. subtilis globigii,* representing 1.6–2.2 x 10^9 CFU/mL	30 min	99.99% killed	11
	0.05%, pH 11.0, 20°C			50% spores survived	
Hydrogen peroxide (H_2O_2)	25.8%, 24°C	*B. subtilis globigii* spore suspension (no concentration)	15 min	0.001% survived	12
	25.8%, 76°C		<1 min	<0.0001% survived	
	0.88 mol/L, pH 5.0	10^6 CFU/mL *B. subtilis* spore suspension	3 h	100% killed	13
	0.88 mol/L, pH 4.3	10 mL *B. subtilis* spore suspension coated onto stainless steel carriers	6 h	100% killed	
Peracetic acid (CH_3COOOH)	0.13 mol/L, pH 5.0, 6.5, 8.0	10^6 CFU/mL *B. subtilis*	<30 min	100% killed	13
	0.39 mol/L, pH 4.0, 7.0, 9.0	10 mL *B. subtilis* spore suspension coated on stainless steel carriers	24 h	100% killed	
Formaldehyde (CH_2O)	4% in water	10^8/mL *B. anthracis*	2 h	10^4 inactivation factor	14
	400 mg/m^3, 30% RH	10^2–3 x 10^8 *B. globigii* NCTC 10073 dried on disks	22 min	1 log_{10} reduction, at 23.5°C–25°C	15
	280 mg/m^3, 50%RH		31 min		
	250 mg/m^3, 80% RH		16 min		
	400 mg/m^3, 98% RH		9 min		
Glutaraldehyde ($C_5H_8O_2$)	2% in water, pH 8.0	10^8/mL spores *B. anthracis*	15 min	10^4 inactivation factor	14
Sodium hydroxide (NaOH)	5%, 27.8ºC	7 x 10^9 spores/mL *B. subtilis*	1.5 h	99% killed	16
	5%, 21.1ºC		3.6 h	99% killed	
Gaseous sterilization					
Ethylene oxide (C_2H_4O)	Exposed to constant boiling HCL at 20°C for 30 min before exposure to ethylene oxide at room temperature	*B. globigii* and *B. anthracis* dried onto suture loop carriers (no concentration)	1 h	100% killed	17
	500 mg/L, 30%–50% RH, 54.4°C	~10^6 spores *B. globigii* on nonhygroscopic surfaces	30 min	4-log reduction	18
		~10^6 spores *B. globigii* on hygroscopic surfaces		6-log reduction	
Chlorine dioxide (ClO_2)	40 mg/L, 60%–80% RH, 25°C–27ºC	1.4 x 10^6/0.2 mL *B. subtilis* subsp. *Niger* dried on paper and aluminum foil strips	1 h	100% killed	19
	30 mg/L, 80%–85% RH, 30ºC	10^6 spores/biologic indicator; *B. subtilis* subsp. *Niger*	30 min	100% killed (estimated time to kill 90%, 4.4 min)	20
	6–7 mg/L, 20%–40% RH, 23ºC	10^6 spores/biologic indicator; *B. subtilis* subsp. *Niger*	30 min	10^1 CFU/biologic indicator (estimated time to kill 90%, 4.2 min)	21
	70%–75% RH for 0.5 before exposure, 23ºC		15 min	0 CFU/biologic indicator (estimated time to kill 90%, 1.6 min)	
Hydrogen peroxide (H_2O_2) plasma	0.208 mg/L, 1.5 Torr pretreatment for 10 min; 2.49 MHz, 150 W of pulsed plasma in a cycle of 0.5 ms plasma on, 1.0 ms plasma off	3.4 x 10^5 *B. subtilis* subsp. *globgii* spores on paper disks and packaged in spun-bonded polyethylene	15 min	100% killed	22
Methylene bromide (CH_3Br)	3.4–3.9 g/L, room temperature in the presence of moisture	1 x 10^5–5 x 10^7 spores of *B. anthracis* dried on sterile filter paper strips	24 h	100% killed	23

Table 2. Efficiency of chemicals, gases, and radiation on the inactivation of *Bacillus* spores

Method	Concentration	Inoculum size	Time	Efficiency	Ref.
Peracetic acid vapor (CH_3COOOH)	1 mg/L, 80% RH	6 x 10^5 – 8x 10^5 *B. subtilis niger* dried on filter-paper disks and glass squares	10 min	<1 spore remained on paper and glass	24
	1 mg/L, 60% RH			2 spores remained on paper; 38 spores remained on glass	
	1 mg/L, 40% RH			24 spores remained on paper; 1,530 spores remained on glass	
Propylene oxide (C_3H_6O)	1,250 mg/L, 86% RH, 36°C–38ºC	9.5 x 10^5–1.1 x 10^6 spores *B. subtilis niger* dried on filter paper	1.05 h	90% killed	25
	1,000 mg/L, 37°C	2.5 x 10^7 spores *B. subtilis niger* in cereal flakes	3 h	3.7% survived	
Ozone (O_3)	1.0 mg/L generated in water pH 3	1.8 x 10^5 spores/mL *B. cerus*	5 min	<10^1 CFU/mL survived	26
	3.0 mg/L, preconditioned at 54% RH	10^8–2 x 10^8 *B. subtilis* dried on filter paper	1.5 h 95% RH	<0.001% survived	27
		10^8–2 x 10^8 *B. cerus* dried on filter paper	1.5 h 95% RH	<0.001% survived	
	900 ppm, preconditioned at 65%–70% RH for 15 h	5 x 10^7 spores/glass coupon	30 min 80% RH	10^0 survived	28
			60 min 70% RH	10^0 survived	
Radiation					
UV	85% 2537A	*B. anthracis* (mixed spores and vegetative forms) in beef extract agar pH 7.4 (no concentration)	452 ergs/mm^2	90% killed	29
	4,800 µWs/cm^2	0.1 mL of 10^8 *B. anthracis* spore suspension dried on aluminum carriers	<96 h	2.4 log reduction, unreliable results	30
	450,000 µWs/cm^2	0.1 mL of 10^8 *B. anthracis* spore suspension dried on ceramic carriers	<96 h	2.03 log reduction, unreliable results	
	52.8 x 10^6 µWs/cm^2	0.1 mL of 10^8 *B. anthracis* spore suspension dried on wood carriers	30 h	0.67 log reduction	
Gamma irradiation		10^6 spores/mL *B. anthracis*	Dose of 1 x 10^6 rad	100% killed	31

[a]RH, relative humidity; conversions: 1 ppm = 1 mg/L; mol/L = gram molecular weight/L; 1 rad = 100 ergs/g; and 1 watt = 10^7 ergs/s.

nated with *B. anthracis* spores (40). In this instance, contamination was greatly reduced immediately after treatment and was undetectable 6 months later. However, the possible role of formaldehyde as a carcinogen has limited its use. Formaldehyde can be neutralized with ammonium bicarbonate after fumigation, reducing its carcinogenic properties.

Gamma radiation was used in the 1960s and 1970s to disinfect *B. anthracis*–contaminated imported bailed goat hair. A study by Horne et al. suggested that a dose of 1.5 megarads from a 200,000-rad/hour cobalt source was sufficient to kill most resistant spores when mixed with goat hair; 2 megarads was recommended to include a margin of safety (31). After the intentional release of *B. anthracis* through the postal system in 2001, pursuing a decontamination method for the undelivered mail was essential. Gamma radiation was used to decontaminate all mail from contaminated facilities on the basis of these data.

Summary

Multiple technologies may be needed to decontaminate buildings and their contents. As in a laboratory, where some items are wiped, some items are autoclaved, and some spaces are treated with gas, more than one method may be required for decontamination. Also, for certain decontamination tasks, e.g., cleaning small heat-proof and water-proof objects, more than one option will be available. Further, even within the context of one type of application (e.g., walls; ducts for heating, ventilating, air conditioning, and refrigeration; carpet; and small objects), potentially conflicting priorities exist between bioefficacy, logistics, and safety.

Our review suggests two conclusions. First, additional scientific research is needed. Although transferring the methods used to decontaminate or sterilize laboratory or food industry settings to decontaminating buildings may be useful, this transfer of methods has not been scientifically tested. Also, much of the data available is based on other *Bacillus* species; more testing with or correlation to

B. anthracis contamination is suggested. Second, choosing between technologies is a complex issue, and a formal decision process would be useful. Various parties in the public and private sector have suggested numerous, sometimes disparate, methods for the inactivation of *B. anthracis* spores in contaminated environments. Further research is needed regarding improved methods for remediation of environments contaminated with *B. anthracis* spores, and the literature summarized here provides a basis for that effort.

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Children with Respiratory Disease Associated with Metapneumovirus in Hong Kong

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Human metapneumovirus (HMPV) is a newly discovered pathogen thought to be associated with respiratory disease. We report the results of a study of 587 children hospitalized with respiratory infection over a 13-month period. HMPV was detected in the nasopharyngeal aspirates from 32 (5.5%) children by reverse transcription-polymerase chain reaction. HMPV infection was associated with clinical diagnoses of pneumonia (36%), asthma exacerbation (23%), or acute bronchiolitis (10%). When compared to those with respiratory syncytial virus infection, children with HMPV infection were older, and wheezing was more likely to represent asthma exacerbation rather than acute bronchiolitis. HMPV viral activity peaked during the spring-summer period in Hong Kong. Phylogenetically, all HMPV virus strains from Hong Kong belonged to one of the two genetic lineages previously described. HMPV contributed to 441.6 hospital admissions per 100,000 population <6 years of age.

Globally, respiratory infections in childhood are a leading cause of disease, contributing to absenteeism and economic strain through use of healthcare resources (1). In the developing world, respiratory infections are also a major cause of childhood death, although the contribution of viruses to such deaths is unclear (2). Respiratory syncytial virus (RSV) and influenza are recognized as important contributors to hospitalization (3–5). Despite sensitive diagnostic methods, an etiologic agent still cannot be identified in a portion of children with acute respiratory infection (6). Human metapneumovirus (HMPV) is a recently discovered respiratory virus belonging to the family *Paramyxovidiridae* (7,8), and its clinical significance is still being defined. After its initial discovery in the Netherlands, HMPV has been detected in respiratory specimens from patients of all ages in a number of countries, e.g., Canada, Australia, United Kingdom, and Finland (9–14). In children, HMPV has been reported to cause disease similar to that of RSV; signs and symptoms range from severe cough to bronchiolitis and pneumonia. However, a detailed analysis of clinical signs in children is lacking. Furthermore, HMPV has not been reported in tropical or subtropical regions. Routine immunization of children against influenza is now under active consideration by the Advisory Committee on Immunization Practices, and vaccines for RSV are being developed. Therefore, defining the role of the newly emerging HMPV is important in childhood respiratory disease.

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Materials and Methods

Study Design

The Hong Kong Special Administrative Region is located within the tropics but has a subtropical climate. Within this region of Hong Kong Island are 288,371 persons ≤18 years of age and 84,018 ≤6 years of age (based on 2001 Census data). Two publicly funded hospitals of the Hospital Authority of Hong Kong, Queen Mary Hospital and Pamela Youde Nethersole Eastern Hospital, provide 90% of all acute pediatric hospital care (admission ratio 1:1.65). At Queen Mary Hospital, a nasopharyngeal aspirate is routinely collected for viral investigation from all children hospitalized with acute respiratory disease.

We investigated a systematic sample of children (≤18 years of age) admitted with acute respiratory infection to Queen Mary Hospital during a 13-month period. From August 2001 to March 2002, all children admitted to Queen Mary Hospital with symptoms of respiratory infection on one fixed day each week were included in this study of HMPV infection. From April through August 2002, study enrollment was increased to twice weekly. The results of virologic diagnosis of HMPV were not available to the attending pediatricians. The clinical features of children identified to have HMPV infection were compared with age-matched controls with influenza A or RSV infection.

Viral Diagnosis

Nasopharyngeal aspirates from patients were tested for RSV, influenza A and B, adenovirus, and parainfluenza types 1, 2, and 3 by culture and immunofluorescent antigen

detection as previously described (15,16). An aliquot of these aspirates was snap-frozen for testing for HMPV. Viral RNA were extracted by using the RNAeasy kit (QIAGEN GmbH, Hilden, Germany) and tested by reverse transcription-polymerase chain reaction (RT-PCR) with primers to the viral L gene as previously described (7). All positive results were confirmed by retesting with a nested RT-PCR with primers to regions of the M gene conserved between HMPV and avian pneumovirus (8).

Twelve microliters of viral RNA was amplified in 20 µL volumes containing the following components: 2 mM Tris-HCL (pH7.5), 10 mM NaCL, 0.01 mM EDTA, 0.11 mM DTT, 0.001% NP-40, 0.5 mM each of the four deoxynucleotide triphosphates, 7.5 ng random primers (Invitrogen, Life Technology, Carlsbad, CA), and 20 U of Superscript II Rnase H^- Reverse Transcriptase (Invitrogen, Life Technology). The reactions were allowed to proceed in a thermocycler programmed to incubate for 50 min at 42°C and 3 min at 94°C cycler (Perkin-Elmer Cetus, Gouda, the Netherlands). Five microliters of cDNA was used for PCR amplification reaction with 0.5 µM of two primers (sense 5′-CATGCCCACTATAAAAGGTCAG-3′ and anti-sense 5′-CACCCCAGTCTTTCTTGAAA-3′), corresponding to the sequence of the L gene of metapneumovirus. Samples were amplified by heating at 95°C for 12 min, 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final of extension period of 7 min at 72°C. Similarly, 5 µL of cDNA was used for a first PCR amplification reaction with 0.5 µM of two degenerated primers (sense 5′-AARGTSAATGCATCAGC-3′ and anti-sense 5′-CAKATTYTGCTTATGCTTTC-3′), corresponding to the sequence of the matrix gene of metapneumovirus, which can detect both avian and human metapneumovirus. Samples were amplified in similar reaction mixture previously described by heating at 95°C for 12 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and a final of extension period of 7 min at 72°C. A 5-µL aliquot of the first PCR product was transferred into a second PCR tube for nested PCR by using two inner specific M gene primers for HMPV (5′-ACACCTGTTACAATACCAGC-3′ and 5′-GACTTGAGTCCCAGCTCCA-3′). The reaction mixture was subjected to a further 95°C, 12 min, 30 cycles of 94°C, 55°C, and 72°C for 1 min each, and a final of extension period of 7 min at 72°C. PCR products were analyzed by electrophoresis in a 2% (w/v) agarose gel and stained with 0.5 µg/mL of ethidium bromide. The sizes of L gene and M gene nested PCR products were 171 bp and 201 bp, respectively. Only specimens with a positive result for both tests were regarded as confirmed positive.

The 171-bp fragment of the RT-PCR–amplified product of the viral L gene was sequenced for phylogenetic analysis. The PCR-amplified DNA was sequenced by using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). Briefly, PCR products were purified by using the QIA quick PCR Purification Kit (QIAGEN GmbH) according to manufacturer's instruction. A total of 35 to 50 ng of purified PCR products were mixed with two tubes containing 1.6 pmol (forward and reverse), 4 µL Terminator reaction mix (containing deoxy- and dideoxy-nucleotides, and modified Taq polymerase), and made up to a final volume of 10 µL with MilliQ water. Cycle sequencing was performed in the thermocyler with profile consisted of a 96°C denaturation step for 30 s, followed by an annealing temperature of 50°C for 15 s and extension temperature of 60°C for 4 min, for a total of 25 cycles. Unincorporated dye terminators and nucleotides were removed by using the DyeEx kit (QIAGEN GmbH). The procedure was performed according to the user manual of the package. The DNA template was denatured at 94°C for 4 min and stored in ice, ready for sequencing on a Perkin-Elmer 377 XL DNA sequencer (Applied Biosystems). DNA sequences were aligned by using the Clustal X software, and phylogenetic analysis was conducted by using MEGA (v. 2.1, Arizona State University, Temple, AZ). The distances for the multiple aligned DNA sequences were estimated by using the Jukes-Cantor method and a phylogenetic tree constructed by using neighbor-joining method. The tree was subjected to bootstrap test (100 replicates) and bootstrap values $\geq$40 are shown.

Chart and Radiographic Review

All medical records were reviewed by a pediatrician (S.S.C.). At the end of the study, the chest radiographs were reviewed by a pediatric radiologist (P.L.K.), who knew that these children had a cough and febrile illness but did not know the clinical or microbiologic diagnoses.

Results

Epidemiology

Since obtaining an nasopharyngeal aspirates to test for common respiratory viruses is a routine diagnostic procedure, all children with acute respiratory symptoms admitted on the study days were enrolled. A total of 587 patients were studied; 302 were enrolled in the first 7 months of the study with patients being admitted on one fixed day of each week. Another 285 patients were enrolled during the last 6 months, with patients admitted on two fixed days of the week recruited to the study. The study sample represented 15.5% of all 3,787 acute pediatric admissions to Queen Mary Hospital and 22.9% of all 2,563 admissions for acute respiratory disease. Thirty-two (5.5%) children had HMPV virus RNA detected in the nasopharyngeal aspirate specimen when tested by RT-PCR. In comparison, routine immunofluorescent antigen detection and viral cul-

ture documented that 8% of children had RSV (mean age 19.50 ± 17.24 months), 8% had influenza A or B (mean age 30.23 ± 22.61 months), 5% had parainfluenza (mean age 37.75 ± 36.87 months), and 3.1% had adenovirus infections (mean age 44.11 ± 53.22 months). Children with HMPV ranged from 3 months to 72 months of age (mean 31.7 ± 18.7 months) and were older than those with RSV infection (p=0.004). The preponderance of males (M:F 3:1) with HMPV was greater than that of the study group overall (M:F 1.2:1) (p=0.03). The peak of HMPV activity occurred in spring and the early summer months in 2001–02 in Hong Kong (Figure 1). The seasonality of other common respiratory viruses during the study period based on viral diagnoses of all children admitted with respiratory infections to Queen Mary Hospital showed that RSV had a similar seasonality as HMPV (Figure 2). Two children with HMPV disease had co-infection: one with adenovirus and another with influenza A. No other children had a viral co-infection.

During the first 12 months of the study, 2,563 persons were admitted for acute respiratory infections to Queen Mary Hospital. From the monthly HMPV isolation rate in our representative sample, we calculated the total of HMPV infections admitted to Queen Mary Hospital during each of the first 12 study months. We estimated that 126 children were hospitalized with HMPV during this period. On average, the ratio of acute pediatric admissions between Queen Mary Hospital and Pamela Youde Nethersole Eastern Hospital is 1:1.65. Since these two hospitals provide 90% of all hospital admissions for Hong Kong Island, we estimated that 371 persons were admitted to the hospital with HMPV of 84,018 children ≤6 years of age living in Hong Kong Island, resulting in a hospitalization rate of 441.6 per 100,000 population ≤6 years of age. As the mean duration of hospitalization per episode was 3.17 days (Table 1), we estimated HMPV accounted for 1,176 days of hospitalization per year in children ≤6 years of age on Hong Kong Island.

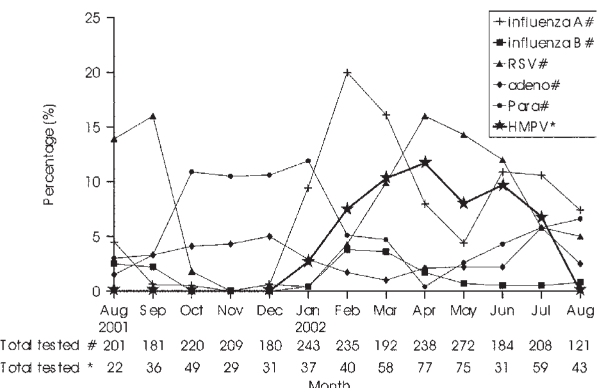

Figure 1. Number of patients tested and percentage positive for human metapneumovirus and the seasonality of other common respiratory viruses during the study period. Data for human metapneumovirus are based on a subset of patients admitted to Queen Mary Hospital (see Methods); data for the other respiratory viruses are based on all children admitted.

Clinical and Laboratory Findings

All children (100%) with HMPV had a fever, and 28 (90%) had cough with sputum (Table 1 and 2). Two children with HMPV had hoarseness without stridor. Five (16.1%) children had febrile seizures; two had three seizures each. Four (12.9%) children with HMPV had a truncal rash (on the chest only in three) that was blanchable, nonpruritic, maculopapular, and transient, lasting for a few hours to a day. Two children had diarrhea not related to antibiotics. Nine (29%) children had lymphopenia (mean 0.96 ± 0.26 x 10^9/L), and two children had elevated transaminases (ALT, AST, and GGT for the two children were 80 MU/L, 40 MU/L, 77 MU/L, and 339 MU/L, 432 MU/L, 634 MU/L, respectively). Of children with HMPV infection, 26.3% had one or more adult family contacts who also had an acute respiratory illness (Table 1).

A 72-month-old child with newly diagnosed acute lymphoblastic leukemia had documented HMPV infection. She had very mild coryzal symptoms for 4 days with a temperature of 38.3°C for a day; and she recovered uneventfully. Fever, coryza, and diarrhea associated with HMPV developed in one other child hospitalized for failure to thrive 6 days after sharing a room for 24 hours with a child with fever and diarrhea, who was subsequently diagnosed to have HMPV infection. This incident suggested a nosocomial transmission with an incubation period for HMPV disease of 5 to 6 days.

The clinical characteristics of patients with HMPV were compared with those of age-matched children with RSV or influenza A (Table 2). Children infected with HMPV tended to have a longer duration of fever than those with RSV, although this finding did not attain statistical significance. In comparison to RSV and influenza, patients with HMPV tended to have a longer hospital stay. However, rapid diagnostic test results for patients with influenza and RSV might influence their quicker discharge from hospital (15). Both HMPV and RSV infections were more likely to be associated with wheezing than were influenza infections. However, in contrast to RSV infection, the cause of wheezing in HPMV infection was often asthma exacerbation rather than acute bronchiolitis. Asthma exacerbation accounted for 66.7% of the wheezing of HMPV-infected children but only 16.7% in RSV-infected children. HMPV was at least as important as influenza as a cause of febrile seizures.

Radiographic Findings

Children with HMPV were more likely to have requests for a chest x-ray (Table 2). Independent of specialist radi-

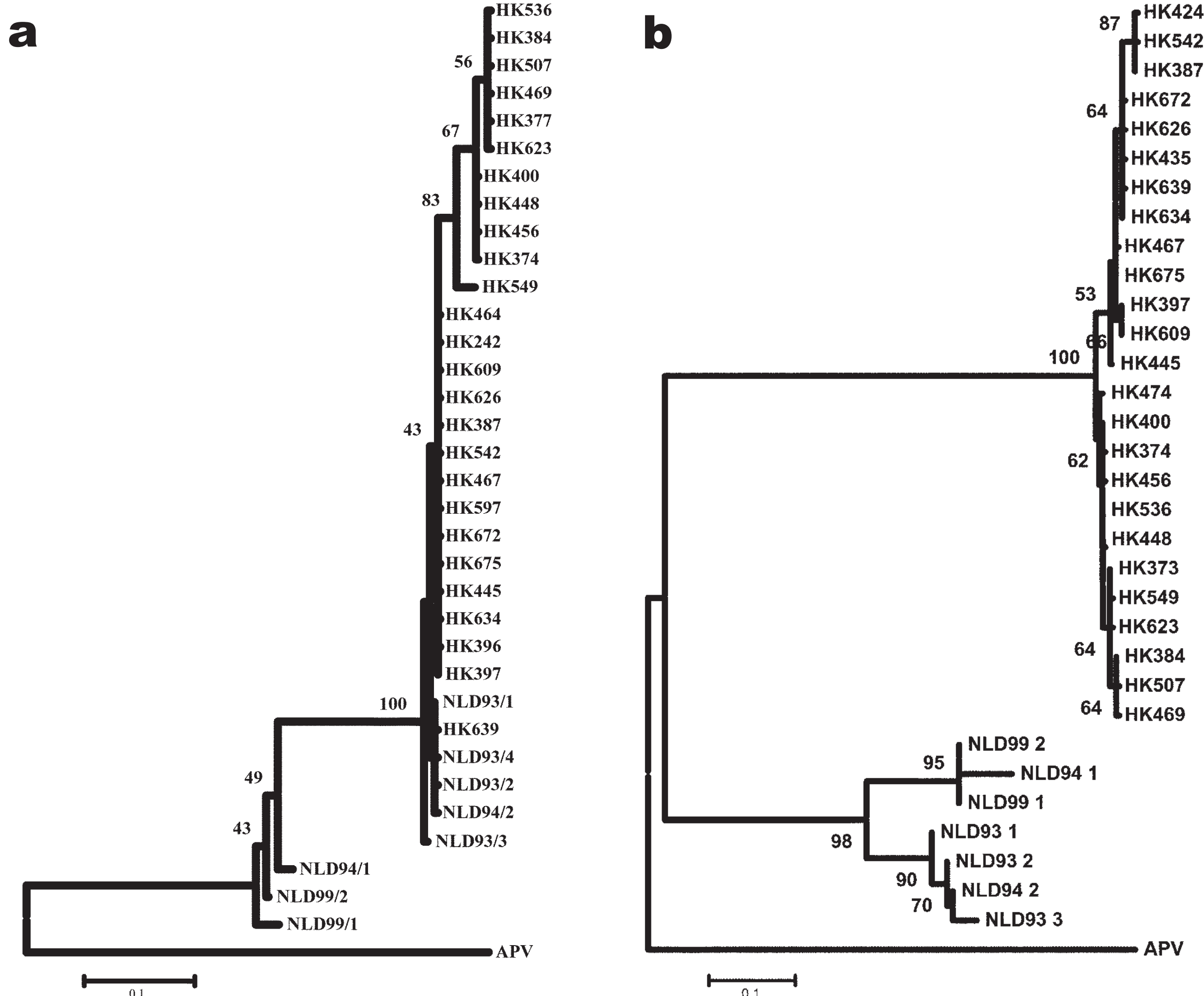

Figure 2. Phylogenetic tree of the human metapneumovirus a) L gene and b) F gene. Viruses detected in Hong Kong are prefixed HK, and the sequences are deposited in GenBank under accession numbers AY294849 through AY294870. Other viral sequences were obtained from GenBank. Abbreviations used: APV, avian pneumovirus; NL, the Netherlands.

ologic assessment, the attending pediatricians diagnosed bacterial pneumonia in four, atypical pneumonia in five, and viral pneumonia in three children, respectively. All chest x-rays were subsequently reviewed by the pediatric radiologist. Perihilar peribronchial thickening, perihilar patchy consolidation, or both were found in 14 patients, suggesting a viral infection. Viral or atypical pneumonia was diagnosed in one child, and viral or bacterial pneumonia was diagnosed in two. Hyperinflation was seen in five children. No child had lobar consolidation.

Phylogenetic Data

Previous studies have shown two distinct phylogenetic lineages of HMPV (8). The viruses detected in Hong Kong during 2002 belonged to one of these lineages (Figure 2). The viruses from the patients with presumed nosocomial transmission were genetically identical.

Discussion

In a representative sample of children hospitalized with acute respiratory symptoms during a 13-month period, we found HMPV virus activity in the spring and summer months. Previous studies, all from temperate regions, have reported HMPV to be a virus with a winter-spring seasonality. In contrast to temperate regions, RSV (and sometimes influenza) in Hong Kong also has a spring-summer seasonality. Surveillance over more years is needed to establish whether the seasonality of HMPV is a recurrent pattern. Our estimate of 441.6 HMPV-associated hospitalizations per 100,000 children ≤6 years of age annually can be compared with recent estimates of influenza-related hospitalization in Hong Kong, which ranged from 2,882 per 100,000 in children <1 year of age to 773 per 100,000 children 2–5 years of age (5).

Table 1. Fever and duration of hospitalization of 32 children admitted with HMPV with age-matched controls with RSV and influenza A

Characteristics	HMPV Mean (SD)	RSV Mean (SD)	Influenza A Mean (SD)	p value[b]
Age (months)	31.70 (18.40)	31.75 (18.41)	31.25 (18.57)	0.99
Duration of hospitalization (days)	3.17 (1.39)[c]	2.81 (1.18)	2.44 (0.96)	0.054
Highest temperature in hospital (°C)[c]	39.20 (0.59)	39.04 (0.54)	39.23 (0.60)	0.44
Duration of fever	4.53 (2.23)	3.57 (1.83)	4.50 (1.86)	0.12

[a]HMPV, human metapneumovirus; RSV, respiratory syncytial virus.

[b]p value performed using the analysis of variance (ANOVA) test.

[c]Two children with HMPV infection were excluded from analysis. A 5-year-old girl newly diagnosed with acute lymphoblastic leukemia had coryzal symptoms and was febrile with nasopharyngeal aspirates positive for HMPV on day 2 of admission. Her total hospitalization stay for chemotherapy and *Pseudomonas* septicemia was 42 days. A 3-month-old girl was admitted for evaluation of failure to thrive and to gain weight; fever, coryza, and diarrhea developed on day 7 of hospitalization. Her total hospitalization of 10 days was also removed from analysis.

[d]This excluded children who were not febrile in the hospital: three children with HMPV, seven with RSV, and four with influenza A.

From this preliminary report, HMPV appears to be an important respiratory pathogen in children, causing a wider spectrum of disease than previously appreciated. Nine of the 32 patients with HMPV had wheezing, asthma exacerbation, or bronchiolitis as a symptom. Approximately one third of patients with HMPV infection were clinically diagnosed to have pneumonia. Not all patients had chest x-rays. However, 17 of the 25 patients who did had abnormalities in their chest x-rays. Thus, a minimum estimate for radiologic abnormalities in children hospitalized with HMPV infection was 53%. Influenza has been previously reported to be a major cause of febrile seizures (17). We found that HMPV may also be an important cause of febrile seizures. In fact, some of the children had multiple seizures during the same episode of HMPV infection. Association of HMPV with febrile seizures, rash, diarrhea, and transaminases has not been previously reported. HMPV was previously reported to cause severe lower respiratory disease and death in children with hematologic malignancies (12,14). We documented HMPV in a child with acute lymphoblastic leukemia, but the illness was mild and self-limited, possibly because infection occurred at the time of diagnosis, before any immunosuppressive therapy was initiated.

Over half of the patients with HMPV had an influenza-like illness reported in one or more of their family contacts (all ages), while 26% reported an adult family member with an influenzalike illness. Seroepidemiologic data in the Netherlands showed that all children are seropositive for HMPV antibody by 10 years of age (7). Further virologic studies of family contacts may elucidate the role of household transmission of HMPV. Recurrent infection has been documented in a few children, and the virus has also been detected in adults (7,9,11,12,14).

When compared to age-matched children infected with RSV or influenza, a greater proportion of children with

Table 2. Characteristics of 32 children admitted with HMPV compared with age-matched controls with RSV and influenza A[a]

	HMPV	RSV	Influenza A	p value[b]		
Characteristics	No. positive/ total (%)	No. positive/ total (%)	No. positive/ total (%)	Overall	HMPV vs. RSV	HMPV vs. influenza A
Influenzalike illness in family contact	10/19 (52.6)	7/29 (24.1)	19/24 (79.1)	0.0003	0.29	0.37
Influenzalike illness in adult family contact	5/19 (26.3)	4/29 (13.8)	13/24 (54.2)	0.005	0.68	0.45
Febrile seizures	5/32 (15.6)	1/32 (3.1)	3/32 (9.4)	0.229	—	—
Congested pharynx	12/32 (37.5)	11/32 (34.4)	11/32 (34.4)	0.955	—	—
Rash	4/32 (12.5)	1/32 (3.1)	4/32 (12.5)	0.331	—	—
Enlarged liver	2/32 (6.3)	0/32 (0.0)	4/32 (12.5)	0.331	—	—
Otitis media	4/32 (12.5)	0/32 (0.0)	0/32 (0.0)	0.201	—	—
Diarrhea	2/32 (6.3)	1/32 (3.1)	3/32 (9.4)	0.586	—	—
Crepitations	18/32 (56.3)	14/32 (43.8)	3/32 (9.4)	0.0003	0.50	0.0007
Wheezing	9/32 (28.1)	12/32 (37.5)	2/32 (6.3)	0.0109	0.60	0.04
Asthma exacerbation	6/32 (18.8)	2/32 (6.3)	2/32 (6.3)	0.167	—	—
Acute bronchiolitis	3/32 (9.4)	10/32 (31.3)	0/32 (0.0)	0.0009	0.13	0.37
Pneumonia	12/32 (37.5)	5/32 (15.6)	1/32 (3.1)	0.0017	0.30	0.009
Abnormal chest x-ray	17/25 (68.0)	11/18 (61.1)	1/17 (5.9)	0.0002	0.89	0.0002
Lymphopenia ($\leq$1.5 x 10^9/L)	9/31 (29.0)	2/27 (7.4)	12/29 (41.4)	0.017	0.34	1.0
Neutropenia (ANC<1 x 10^9/L)	2/31 (6.5)	0/27 (0.0)	4/29 (13.8)	0.125	—	—
Elevated transaminases	2/15 (13.3)	0/5 (0.0)	3/11 (27.3)	0.357	—	—

[a]HMPV, human metapneumovirus; RSV, respiratory syncytial virus.

[b]p values performed using McNemar chi-square test.

HMPV infection had lower respiratory tract involvement leading to more chest x-rays being performed. HMPV appeared to be a stronger trigger for asthma exacerbation than RSV or influenza (Table 2). In a recent study of children hospitalized with wheezing in Finland, HMPV was detected in 10 (32%) of 31 children recruited during the period of peak HMPV activity (January–April) or in 7.5% of the 132 children overall (13). The chemokine profile (interleukin 8 and RANTES) in nasal secretions of children infected with HMPV was different from that reported in infections with RSV.

The role of viral respiratory tract infections in acute and chronic asthma has been a subject of much research interest. Globally, the prevalence of asthma has increased, and recent data show that the prevalence of asthma in Hong Kong children 6–7 years of age is 8% (18). Viruses have been demonstrated to be epidemiologically associated with asthma in at least two ways. They may initiate the development of the atopic state in infants and children. Further, they cause acute exacerbations in children with established asthma. RSV and parainfluenza, with their tendency to cause bronchiolitis in infants, have been most intensively studied (19). Recently rhinoviruses have also been recognized as a principal trigger of asthma exacerbation in older children and adults (20). Our data indicate that HMPV may also be important in this regard, and its role in the pathogenesis of wheezing in the younger child, as well as the mechanism by which it produces asthma symptoms, warrants further study. Elucidation of the roles of cytokines and chemokines in asthma attacks associated with viral infections, such as HMPV, will have implications for the rational use of anti-inflammatory agents such as corticosteroids and leukotriene receptor antagonists.

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Human Metapneumovirus Infections in Hospitalized Children[1]

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We evaluated the percentage of hospitalizations for acute respiratory tract infections in children <3 years of age attributable to human metapneumovirus (HMPV) and other respiratory viruses in a prospective study during winter and spring 2002. We used real-time polymerase chain assays and other conventional diagnostic methods to detect HMPV, human respiratory syncytial virus (HRSV), and influenza viruses in nasopharyngeal aspirates of children. HMPV was detected in 12 (6%) of the 208 children hospitalized for acute respiratory tract infections, HRSV in 118 (57%), and influenza A in 49 (24%). Bronchiolitis was diagnosed in 8 (68%) and pneumonitis in 2 (17%) of HMPV-infected children; of those with HRSV infection, bronchiolitis was diagnosed in 99 (84%) and pneumonitis in 30 (25%). None of the HMPV-infected children was admitted to an intensive-care unit, whereas 15% of those with HRSV or influenza A infections were admitted. HMPV is an important cause of illness in young children with a similar, although less severe, clinical presentation to that of HRSV.

The human metapneumovirus (HMPV) is the first member of the new *Metapneumovirus* genus (*Paramyxoviridae* family) that infects humans (1,2). The human respiratory syncytial virus (HRSV) belongs to a separate genus within the same family (3). HMPV has been recently identified in nasopharyngeal aspirates of children and adults with acute respiratory tract infections (ARTI) in various parts of the world (1,4–7). The clinical syndrome of the infected children ranges from mild respiratory problems to bronchiolitis and pneumonitis (1,7–9).

When reverse-transcription polymerase chain reaction (RT-PCR) has been used, the proportion of HMPV detected in nasopharyngeal aspirate samples from children with unexplained ARTI has varied from 1.5% to 10% (1,4,7). However, most retrospective studies had limitations: for example, they were small, excluded patients who tested positive for other viruses, only superficially described the clinical features of the disease, and lacked data on illness severity and death. Moreover, in the absence of a control group, these studies could not differentiate whether HMPV was a colonizing or a pathogenic virus. More recently, Stockton et al. identified HMPV RNA in 2.2% of 405 specimens from patients with influenzalike illnesses who consulted general practitioners in England, although few swabs were collected from children <5 years of age (6).

The objectives of this study were to estimate the relative contribution of HMPV in children's hospitalization for ARTI and to define its clinical features and seasonal pattern relative to other common respiratory viruses over a single winter season.

Materials and Methods

Study Design

Participants were children ≤3 years of age who were hospitalized from December 15, 2001, to April 20, 2002, at Laval University Hospital Center in Québec City, Québec, Canada. Case-patients were children admitted for an ARTI (mostly bronchiolitis, pneumonitis, and laryngotracheobronchitis) who had a nasopharyngeal aspirate collected as part of the investigation of their illness (in this hospital, collecting such samples is standard practice to assess the presence of HRSV in children). The research nurse at the microbiology laboratory that received the nasopharyngeal aspirate specimens identified eligible case-patients. Case-patients hospitalized twice were counted as two cases. A specific questionnaire for the study was completed at admission by a single research nurse with the parents. At the end of the hospitalization, the children's charts were reviewed to collect clinical and laboratory data by using a standardized protocol. Eligible controls were children hospitalized for any elective surgery who had no respiratory symptoms or fever. At admission, the nurse obtained a signed consent from parents and collected a nasopharyngeal aspirate (1–2 mL). The study was

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approved by the Centre Hospitalier Universitaire de Québec research ethics board.

Laboratory Testing

For this study, all specimens from case-patients and controls were tested by RT-PCR for HMPV, influenza A and B, and HRSV. Antigen detection for HRSV was performed for all case-patients immediately at admission. Viral cultures and other antigen detection assays were performed on request of the treating physician. The rest of the specimen was then frozen at –80°C until subsequent RT-PCR studies.

RNA Extraction and RT-PCR Studies

Viral RNA was extracted from 200 µL of nasopharyngeal aspirate specimens by using the QIAamp viral RNA Mini Kit (QIAGEN, Inc., Mississauga, ON, Canada). Complementary cDNA was synthesized by using 10 µL of eluted RNA and the Omniscript Reverse Transcriptase (QIAGEN). Random hexamer primers (Amersham Pharmacia Biotech, Baie d'Urfé, Québec, Canada) were used in the RT step of all PCR assays, except for HMPV, in which a specific primer (5′-TGGGACAAGTGAAAATGTC-3′) served to synthesize HMPV cDNA. PCR assays were designed to amplify conserved regions of influenza A (10), influenza B (11), and HRSV (12) genes. New PCR primers were designed for amplification of the HMPV N (nucleoprotein) gene. The sequences of the forward and reverse primers were respectively 5′-GAGTCTCAGTACACAATTAA-3′ and 5′-GCATTTCCGAGAACAACAC-3′. Complementary DNA was amplified for all respiratory viruses by using a standardized RT-PCR protocol with the LC Faststart DNA Master SYBR Green 1 Kit (Roche Diagnostics, Laval, Québec, Canada) in a LightCycler instrument (Roche Diagnostics). The melting curve analysis program of the LightCycler was used to identify specific PCR products. Each PCR assay could detect at least 50 copies of viral target. For phylogenetic studies, nucleotide sequences were determined from amplified HMPV F (fusion) gene products, then analyzed by using the neighbor-joining algorithm and Kimura-2 parameters (9).

Standard Viral Cultures and Antigenic Assays

Specimens were injected onto 96-well plates containing 10 cell lines (MDCK, LLC-MK2, Hep-2, human foreskin fibroblast, Vero, mink lung, A-549, rhabdomyosarcoma, 293, and HT-29) and then incubated for 21 days. A positive cytopathic effect was confirmed by immunofluorescence testing with monoclonal antibodies or by RT-PCR (HMPV) (9). Detection of HRSV and influenza antigens was performed directly on nasopharyngeal aspirate samples by using commercially available immunoenzymatic assays (RSV TestPack, Abbott Laboratories, Abbott Park, IL; Directigen Flu A + B, Becton Dickinson Microbiology Systems, Sparks, MD). Viral antigens for adenoviruses and parainfluenza viruses 1–3 were sought in specimens by an immunofluorescence method with specific monoclonal antibodies (9).

HMPV in the General Population

To further assess the seasonal distribution, affected age groups, and frequency of HMPV, we compared data from this study with data from the general population using positive viral cultures reported by our regional virology diagnostic laboratory, the only one performing viral cultures for the Québec City area (population 600,000). Isolation of HMPV was achieved by observing typical cytopathic effect on LLC-MK2 cells, followed by PCR confirmation on infected cell culture supernatants (9).

Statistical Analyses

The Wilcoxon nonparametric test was used to compare the age distribution of case-patients and controls and period of hospitalization. The proportion of cases and controls with HMPV infections and the clinical features of children infected with HMPV versus those infected with other respiratory viruses were compared by the chi-square test or the Fisher exact test. Analyses were performed by using SAS software version 8.02 (SAS Institute, Inc., Cary, NC).

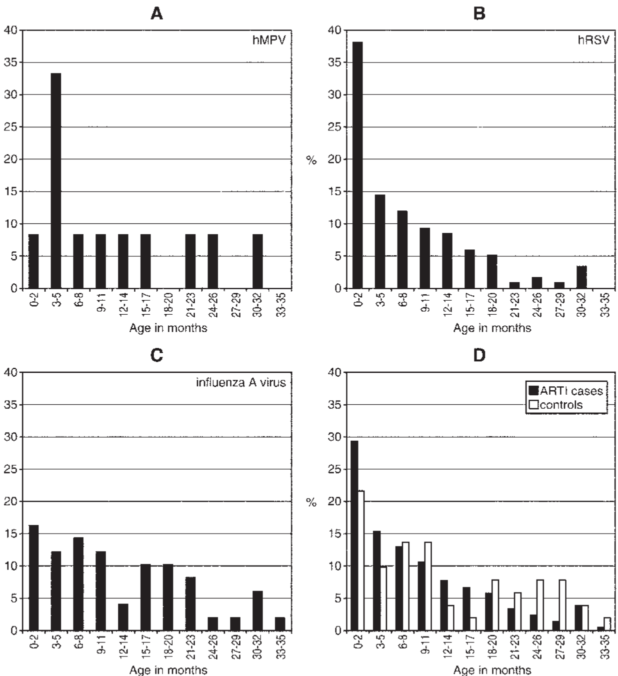

Figure 1. Age at admission of children hospitalized for acute respiratory tract infections caused by human metapneumovirus (HMPV) (A), human respiratory syncytial virus (HRSV) (B), and influenza A (C) as well as for the whole study population (D).

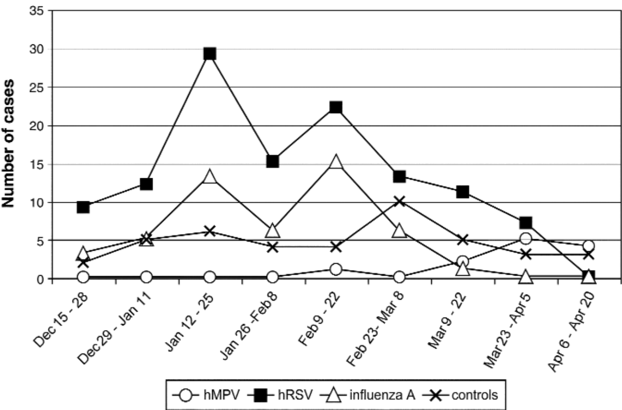

Figure 2. Biweekly distribution of virologically confirmed cases with acute respiratory tract infections and their controls.

Results

Study Population and Viral Etiologic Agents

The study population included 208 hospitalized case-patients with ARTI (including 8 children who were admitted twice) and 51 children who served as controls. The age distribution of case-patients and controls is presented in Figure 1. Infants ≤3 months of age were most likely to be hospitalized, and the rate of hospitalizations steadily decreased in children >3 months. The mean age was slightly younger for case-patients than for controls (mean 9 months vs. 12 months, Wilcoxon test p=0.06). Among cases with ARTI, 56% were male as were 57% of controls (p=0.88). The date of hospitalization was similar for case-patients and controls (p=0.84) (Figure 2). Most children (90%) had no underlying medical conditions at admission.

A nasopharyngeal aspirate sample was taken for all 208 case-patients and 51 controls. For case-patients, the mean delay between the onset of symptoms and collection of nasopharyngeal aspirates was 6 days (median 4 days) (Table 1). This delay did not differ for the different viruses detected. Samples from all 208 case-patients were tested by PCR for HMPV, HRSV, and influenza A and B; 204 samples were tested for HRSV antigen; 172 were assayed for other viral antigens; and 145 were tested by viral culture for the whole panel of respiratory viruses (as ordered by the treating physician). At least one respiratory virus was detected by one of the above methods in 164 (78.8%) cases, whereas none was detected in 44 (21.2%). Combining these diagnostic techniques, 12 cases (5.8%) were positive for HMPV, 118 (56.7%) for HRSV, 49 (23.6%) for influenza A, and none for influenza B (Table 1). In contrast, a virus was not detected by PCR in any of the control samples (p=0.067 for HMPV, one-sided Fisher exact test). PCR testing was not done for adenoviruses and parainfluenza viruses, but these viruses were respectively found in 6/145 (4.1%) and 2/145 (1.3%) of tested case-patients by the use of viral cultures or antigenic assays. Single virus infections occurred in 141 (86.0%) of the 164 positive case-patients, and mixed infection was found in 23 (14.0%). Two of the 12 HMPV infections were mixed (HMPV-influenza and HMPV-HRSV). The other combinations were HRSV–influenza A (18 cases), HRSV-adenovirus (2), and influenza A–adenovirus (1).

Among the 208 case-patients tested by PCR for HMPV, HRSV, and influenza A and B viruses, the positivity rates were 5.8%, 51.0%, 21.6%, and 0%, respectively (Table 1). In addition, 16 other case-patients had one of these four respiratory viruses identified only by culture (one influenza A and one HRSV), only by an antigen detection test (nine HRSV and three influenza A), or by both culture and antigen detection test (two HRSV). Among the eight children who were hospitalized twice, none had the same viral infection at both admissions. The specific combinations observed were HMPV-HRSV (two), HMPV–no virus (one), HRSV–no virus (two), HRSV-influenza (two), no virus–no virus (one).

The biweekly distribution of cases with respiratory tract viruses is shown in Figure 2. HRSV and influenza A infections occurred predominantly from January to March, whereas HMPV infections occurred mostly in March and April. The proportion of children with virologically confirmed respiratory tract infections decreased after February.

Clinical Features of Cases

Given the small number of HMPV cases, the results only suggest trends, as no statistical comparison reached

Table 1. Type of laboratory confirmation by type of infection

	HMPV[a]		HRSV		Influenza A		Adenovirus		PIV 2	
Laboratory test	No. tests	Positive (%)	No. tests	Positive (%)	No. tests	Positive (%)	No. tests	Positive (%)	Test done	Positive (%)
PCR	208	12 (5.8)	208	106 (51.0)	208	45 (21.6)	NA	NA	NA	NA
Culture	145	2 (1.4)	145	37 (25.5)	145	10 (6.9)	145	6 (4.1)	145	1 (0.7%)
Antigen detection	NA	NA	204	94 (46.1)	172	19 (11.0)	81	1 (1.2)	76	1 (1.3%)
Total (+) in at least one test	12 (5.8%)		118 (56.7)		49 (23.6)		6 (4.1)		2 (1.3%)	
Delay between onset of symptoms and NPA, days; mean/median	6.3/5.0		5.2/4.0		8.7/5.0		6.0/6.5		3.0/3.0	

[a]HMPV, human metapneumovirus; HRSV, human respiratory syncytial virus; PIV, parainfluenza virus; NPA, nasopharyngeal aspirate; PCR, polymerase chain reaction; NA, not applicable.

significance. The peak age for hospitalized HMPV infection was 3–5 months, whereas it was 0–2 months for HRSV infection (Figure 1). Influenza A virus infection occurred evenly throughout the first year of life. The peak age for mixed infection was 6–11 months; the frequency of such infections decreased thereafter. Gender was distributed evenly within each virus group, but more males (70%) had mixed viral infections. Most (75% with HMPV, 93% with HRSV, 90% with influenza A virus infection) of the children in the etiologic agent groups had no underlying medical conditions. Three (25%) children with HMPV infection had a cardiac disorder, including one child with multiple medical problems.

Signs and symptoms recorded with the different respiratory viruses were similar (Table 2). The median duration of hospitalization was similar for HMPV, HRSV, and influenza A viruses being respectively 4.5, 5.0, and 4.0 days (p=0.85). Of note, four (33.3%) HMPV-infected children were hospitalized for >7 days, including one child with underlying conditions. None of the children with HMPV infection was admitted to the intensive-care unit (ICU) in contrast to 15% (p=0.22) with HRSV and 16% (p=0.34) with influenza A infections. None of the children in this study died. The duration of the hospitalization for children with no detectable virus was shorter than that for children with single or mixed infection (Wilcoxon test, p <0.001). Two thirds of the children were given antibiotics during their hospitalization, although almost none had specimens collected for bacterial cultures.

At hospital discharge, a final diagnosis of bronchiolitis was given to 67% of children with HMPV, 84% with HRSV, and 51% with influenza A (p <0.001) (Table 3). Otitis media occurred in about half of the children with HMPV, HRSV, and influenza A virus infections. Pneumonitis was less frequently diagnosed in children with HMPV compared to those with HRSV or influenza A (17%, 25%, and 37%; p=0.22). Definitive clinical diagnoses were similar with single and mixed infections.

HMPV in the General Population

The regional virology laboratory received 1,505 respiratory specimens for viral culture from January 1 to June 30, 2002. In total, 36 (including 2 study participants) or 2.9% were positive for HMPV: 24 (67%) in children <2 years of age, 5 (14%) in those 2 to 4 years of age, 4 (11.1%) in adults 30–49 years of age, and 3 (8%) in those ≥70 years of age. No clinical information was available from these cases. Most isolates (81%) were recovered over a 2-month period (from March 23 to May 18). When the seasonal distributions of HMPV in hospitalized children (study population) and in the general population were compared, we found that the study did not cover the entire HMPV season and that it had been stopped just after the peak time of HMPV transmission (April 6–20) (Figure 3).

Phylogenetic Analyses of HMPV Strains

The 12 HMPV strains detected in hospitalized children (study population) clearly clustered into two F lineages as previously reported (1,5,9); nine strains belonged to group 1 (which includes the prototype strain from the Netherlands, GenBank accession no. af371337) and three to group 2 (Figure 4). Seven of the nine group 1 strains had identical F gene sequences although they were not temporally related. At the nucleotide level, similarity between groups was 84% to 85%, compared to 98% to 100% within group 1 and 93% to 99% similarity within group 2, respectively.

Discussion

Our prospective study has shown important clinical and epidemiologic features of HMPV infection. First, our data indicate that HMPV is really a respiratory pathogen with an epidemic behavior. Second, we found that HMPV substantially contributes to ARTI that leads to children's hospitalization, although in smaller proportion than HRSV and influenza viruses. Although all specimens from this study were tested by PCR, only a subset was studied by

Table 2. Signs and symptoms by type of viral infection

Signs and symptoms	% HMPV[a], n=12	% HRSV, n=118	% Influenza A, n=49	% Single virus, n=141	% Multiple viruses, n=23	% No virus detected, n=44	% Total, n=208
Fever	67	57	78	60	74	57	61
Cough	100	99	96	98	100	90	97
Rhinorrhea	92	91	84	87	96	96	90
Retractions	92	95	82	89	96	89	89
Wheezing	83	65	57	59	83	71	64
Lacrymation	25	31	31	33	26	25	30
Diarrhea	8	17	27	17	22	23	19
Vomiting	25	8	10	7	17	2	7
Other	0	26	18	23	17	21	22

[a]HMPV, human metapneumovirus; HRSV, human respiratory syncytial virus. Given the small number of HMPV cases, the results only suggest trends, as no statistical comparison reached significance.

Table 3. Definitive clinical diagnoses by type of viral infection

Complication	% HMPV,[a] n=12	% HRSV, n=118	% Influenza A, n=49	% Single virus, n=141	% Multiple viruses, n=23	% No virus detected, n=44	% Total, n=208
Bronchiolitis	67	84	51	70	83	57	68
Pneumonia	17	25	37	28	30	27	28
Laryngotracheobronchitis	0	10	12	8	17	5	8
Otitis	50	59	55	55	65	55	56
Sinusitis	0	3	6	1	9	2	2
Pharyngitis	0	1	0	1	0	5	2
Flu syndrome	0	2	0	1	0	9	3
Other	8	3	6	6	0	11	7

[a]HMPV, human metapneumovirus; HRSV, human respiratory syncytial virus. Given the small number of HMPV cases, the results only suggest trends, as no statistical comparison reached significance.

viral culture. However, such incomplete virologic testing should not have significantly affected the rate of HMPV infection as evidenced by the absence of additional cases detected by culture. Third, the clinical features associated with HMPV were found to be similar to those of HRSV. Finally, our results suggest that the seasonal pattern of HMPV infection in children may differ from that of HRSV and influenza viruses although additional studies are needed because of our relatively short period of observation.

Recent studies by our group (8,9) and others (1,4,6,7) have suggested that HMPV should be added to the list of human respiratory viral pathogens (13–18) affecting mainly children, but also other age groups as well. Although the difference in HMPV positivity between our 208 case-patients and 51 controls was not statistically significant (p=0.067), the absence of other respiratory viruses such as HRSV and influenza viruses in 83% of the HMPV-infected children and the severity of the symptoms (bronchiolitis, pneumonitis, or both) suggest that HMPV is a pathogenic respiratory virus. The absence of underlying medical conditions in 75% of the HMPV-infected children further illustrates the pathogenicity of HMPV. The use of PCR was particularly advantageous for HMPV detection because this virus is fastidious and difficult to grow in most cell lines (9); in addition, rapid antigenic detection tests are not currently available.

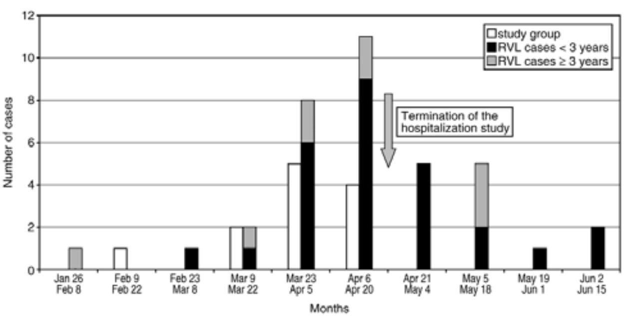

Figure 3. Biweekly distribution of virologically confirmed human metapneumovirus (HMPV) cases from the prospective pediatric study (study group) and from the general population as retrospectively identified in the Québec City Regional Virology Laboratory (RVL).

Our prospective study provides for the first time an estimate of the proportion of ARTI hospitalizations attributable to HMPV in a well-defined pediatric population. From December 15 to April 20, 2002, HMPV was detected in 12 (6%) of 208 children ≤3 years of age who were hospitalized for respiratory tract infections. This probably underestimates the real impact of this virus because our hospitalization study was stopped before the end of HMPV transmission in the community. However, the percentage of hospitalizations caused by HMPV during the study period was much smaller than that attributable to HRSV or influenza A. Our data are comparable to those of a recent small study from Finland in which HMPV was detected in 8% of children (age range, 4 months to 13.5 years) admitted for acute wheezing (7).

Our study found that HMPV disease cannot be distinguished from HRSV and influenza A on clinical findings. However, HMPV disease tended to be somewhat less severe with fewer cases of pneumonia, no admission in the ICU, and a greater proportion of underlying diseases (25%) among infected patients compared with <10% for HRSV or influenza. Nevertheless, HMPV infection was associated with a substantial clinical and economic impact as shown by a median hospital stay of 4.5 days and by the observation that one-third of HMPV-infected case-patients were hospitalized for >7 days.

A small serologic study from the Netherlands showed that all children >5 years of age had HMPV antibodies, which suggests a high level of transmission (1). While our study data were limited to children <3 years of age, they suggest that illness caused by HMPV is greatest in children <2 years of age because they represented 10 (83%) of our 12 hospitalized case-patients and 24 (66%) of the 36 HMPV isolates recovered in our diagnostic virology laboratory. This finding suggests that, similar to other paramyxoviruses such as HRSV, the most severe HMPV infections occur through primary infection in young children. In contrast to HRSV, which peaked during the first 2 months of life, HMPV hospitalizations seem to peak in children at a slightly older age, i.e., between the third and fifth month of life. However, given the small number of HMPV cases, this observation needs to be confirmed in a larger study. Should

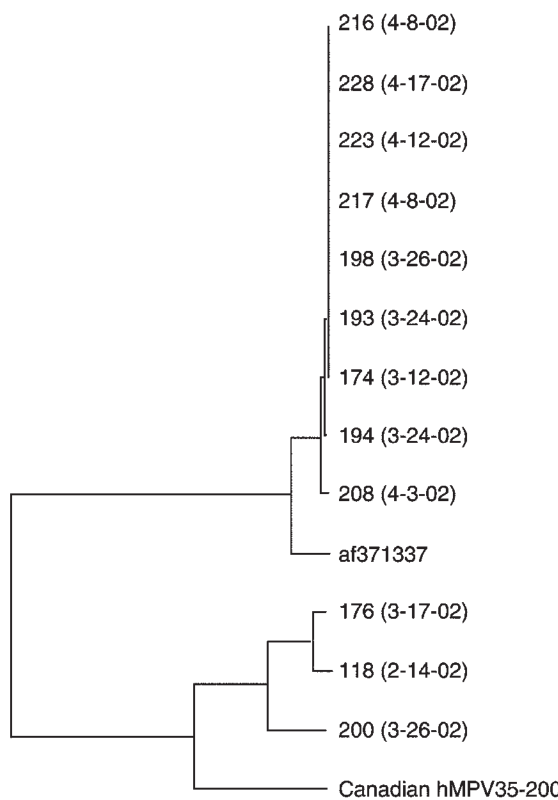

Figure 4. Phylogenetic tree showing sequence analysis of the F (fusion) gene of 12 human metapneumovirus (HMPV) strains detected in 2002 as part of this study and of the prototype strain from the Netherlands (GenBank accession no. af371337) as well as from a Canadian strain (HMPV 35) isolated in 2001.

the same trend be observed, this difference may depend on a longer persistence of maternal antibodies or a less efficient transmission mode in the case of HMPV. Both hypotheses would require additional studies.

During the 4-week period from mid-March to mid-April, HMPV infections clustered (11/12 cases) and were associated with 18.9% of all hospitalizations for ARTI in children at our institution. These findings contrast with those for HRSV and influenza A infections, which occurred mostly in January and February. On the basis of passive surveillance data from our regional virology laboratory, the peak time of HMPV transmission in the community occurred between April 6 and 20, 2002, and continued beyond the conclusion of our study in hospitalized children until the end of May. Although incomplete, such data suggest that seasonal outbreaks of HMPV may differ from those of other common respiratory viruses.

As described for HRSV (12), several strains of HMPV circulated during a very brief period (1 month) in our study area. The HMPV strains segregated into two F subgroups, in agreement with previous studies (1,5,9), although one strain clearly predominated, accounting for 58.3% of all infections. Because of the small number of HMPV strains belonging to one of the F subgroup, we did not attempt to correlate HMPV genotype with clinical outcome. Such viral heterogeneity may allow multiple reinfections throughout life, especially in elderly persons and immunocompromised patients, as we previously reported (8,9).

In conclusion, our study supports the concept of the epidemic nature of HMPV infection and its role as a significant pathogen in severe ARTI of children. Year-long active surveillance studies on consecutive years and in different geographic regions are needed to better define the epidemiology of HMPV.

Acknowledgments

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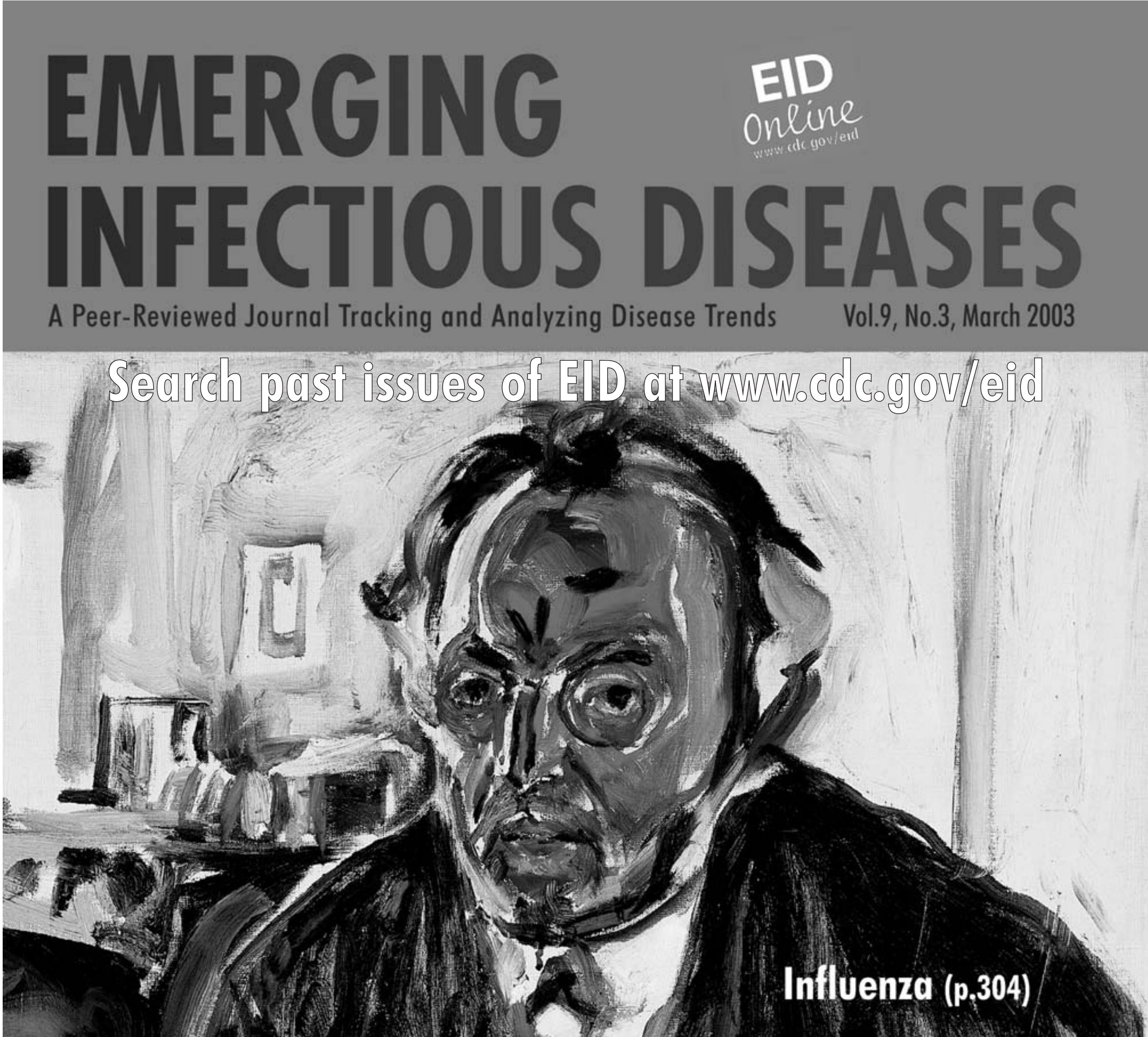

Dead Bird Clusters as an Early Warning System for West Nile Virus Activity

Farzad Mostashari,* Martin Kulldorff,† Jessica J. Hartman,* James R. Miller,* and Varuni Kulasekera*

An early warning system for West Nile virus (WNV) outbreaks could provide a basis for targeted public education and surveillance activities as well as more timely larval and adult mosquito control. We adapted the spatial scan statistic for prospective detection of infectious disease outbreaks, applied the results to data on dead birds reported from New York City in 2000, and reviewed its utility in providing an early warning of WNV activity in 2001. Prospective geographic cluster analysis of dead bird reports may provide early warning of increasing viral activity in birds and mosquitoes, allowing jurisdictions to triage limited mosquito-collection and laboratory resources and more effectively prevent human disease caused by the virus. This adaptation of the scan statistic could also be useful in other infectious disease surveillance systems, including those for bioterrorism.

By the end of 2002, West Nile virus (WNV) activity had been reported in all but four of the lower continental states, with >3,500 human cases reported (1). Since the 1999 WNV outbreak in New York City, which caused thousands of human infections (2) and 59 severe meningoencephalitis cases (3) including 7 deaths, health officials have been searching for an early warning system that could help prevent human illness and deaths. In the summer of 2000, the New York City Department of Health and Mental Hygiene established an unprecedented citywide network of adult mosquito traps, sentinel bird flocks, and system for reporting, collecting, and testing dead birds. Retrospective county-level analysis of year 2000 data showed that dead birds and mosquito pools with laboratory evidence of WNV were collected in the outbreak epicenter, Staten Island, approximately 2 weeks before onset of the first human case (4). However, mosquito and bird collection and laboratory testing are costly and resource intensive.

The county-level density of dead bird (4) and crow (5) reports per square mile was also strongly correlated with levels of WNV activity in 2000. However, county-level analysis is insensitive to small-area clustering. In addition, the density of dead bird reports is confounded by the background bird population, human population density, and the varying propensity of different communities to report dead birds. We describe a method for detecting small-area clustering of dead bird reports above expected levels, present the results of its application to data from 2000, and review its utility in providing an early warning of WNV activity in New York city in 2001.

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Materials and Methods

Data Collection

Data collection procedures were the same for 2000 and 2001 and have been described in detail elsewhere (4,6). In brief, dead birds were reported by the public through an interactive voice-response telephone system or the Internet (Figure 1). The information included the date found and the location and species of the dead bird. A sample of dead birds that met selection criteria (i.e., the bird was recently dead, had little apparent decay or trauma) were submitted for necropsy and testing (4). Since pigeon (Rock Dove) deaths are common but rarely associated with WNV (7,8), they were excluded from all clustering analyses.

Mosquitoes were collected weekly from >100 traps dispersed throughout the city (4). Nearly all areas of the city were within 2 miles of a mosquito trap. Multiple mosquitoes from the same trap and of the same species were pooled, and each pool was tested for evidence of WNV; a result was considered positive if at least one mosquito was infected. The New York City Department of Health and Mental Hygiene conducted citywide active hospital-based physician and laboratory surveillance for human WNV infection (6).

Geocoding

All dead bird reports, mosquito traps, and human case-patients with address information were geocoded to a point location, where possible, with the ArcView Geographic Information System (GIS) software (ESRI, Redlands, CA).

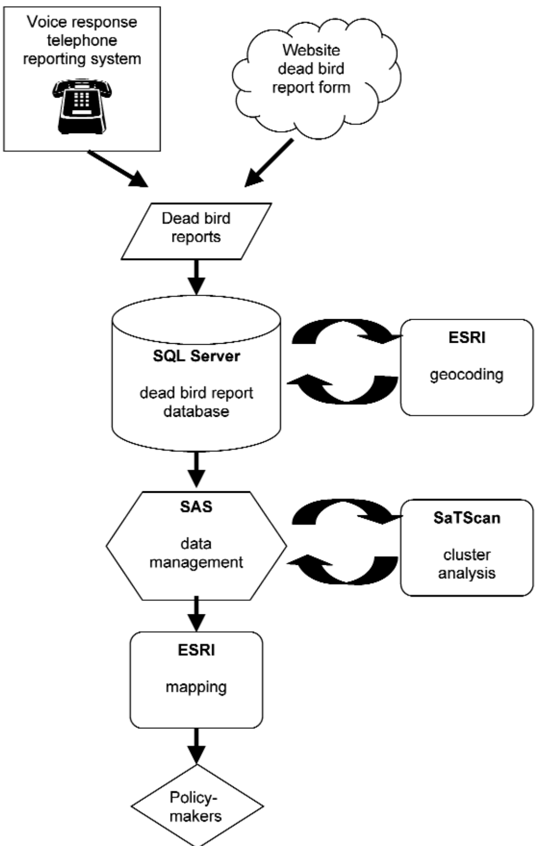

Figure 1. Dead bird cluster surveillance system. SAS, SAS statistical package (SAS Institute, Inc., Cary, NC); ESRI, Environmental Systems Research Institute; SQL, structured query language.

Multiple dead bird reports from the same location on the same date were counted as one. Dead bird reports were attributed to the census tracts (n=2,215; mean area = 0.13 square miles) in which they were found by using a spatial join. The latitude and longitude in decimal degrees for each census tract centroid were used in cluster analyses.

Spatial Scan Statistic

For early detection of localized clusters of dead birds, we used a prospective surveillance system that is based on the spatial scan statistic (9). This scan statistic uses a circular window to represent potential geographic clusters. By continuously changing the circle center and radius, the window scans the geographic area for potential localized clusters without incorporating prior assumptions about their size or location.

Even in the absence of a WNV epidemic, more birds will be reported from some areas than others. To adjust the analysis for such geographic variability, we used historical dead bird counts from a given census tract as baseline (pre-outbreak) controls; recent birds counts were used as cases. We defined cases as the dead bird reports that occurred in the 7 days before the date of analysis. A minimum 2-week buffer zone between case and control birds was also established, thereby limiting the influence of emerging clusters on the analysis. Thus, before laboratory confirmation of a WNV-attributable dead bird cluster, controls were defined as dead bird reports from April 1 until 21 days before the day the analysis was performed. April 1 was chosen as the start date for the control period because many bird species change their geographic location and habitats during the winter months. After laboratory confirmation of WNV from within a cluster area, the control file was frozen to include only birds reported before that cluster, that is, during the pre-outbreak period.

For each circle evaluated for potential clustering with the scan statistic, census tracts are classified as being inside or outside the potential cluster, depending on their centroid location. The number of recently observed dead birds (cases) inside and outside the potential cluster is compared with the expected case-count on the basis of the geographic distribution of historical controls. For example, the expected number of cases inside the circle is equal to the total number of cases citywide, multiplied by the proportion of all dead birds found within the circle during the control period. Based on the observed and the expected, a Bernoulli-based likelihood is calculated for each circle, and the circle with the maximum likelihood is defined as the most unusual cluster, that is, the cluster least likely to be due to chance. To adjust for the multiple testing inherent in the many possible cluster locations and sizes, we evaluated the statistical significance (p value) of this cluster by using Monte Carlo hypothesis testing (9–11). In this method, the likelihood of the most unusual cluster in the observed dataset is rank-ordered among the maximum likelihoods of 999 simulated (randomized) datasets.

We performed the data processing using automated SAS programs (SAS Institute, Inc., Cary, NC) to organize the dead bird reports into case and control files, invoke the spatial scanning software (SaTScan version 2.1, freeware available from: URL: http://www.satscan.org), extract cluster information from the SaTScan output file, archive the cluster data, and export a dbase file for mapping purposes. Using Arcview GIS software, we spatially joined the dbase file to a census tract layer and produced a map displaying the cumulative frequency of dead bird clusters in each census tract.

To test the method, analyses were performed retrospectively for the 2000 data through serial daily replications for every day from June 1 through October 1. To maximize the sensitivity and timeliness of this early warning system, all clusters with $p<0.10$ were mapped. A prospective dead bird clustering surveillance system was implemented in real-time beginning June 22, 2001; daily analyses were performed by using the same definitions of cases and con-

trols as for the year 2000. Maps showing the results for 2000 and 2001 and automated SAS programs for reproducing these analyses on local dead bird reporting data are online (available from: URL: http://www.nyc.gov/health/cluster.html).

Results

Year 2000 Simulation

The first evidence of clustering in New York City in the 2000 simulation was on June 14, when a group of 26 census tracts in northern Staten Island were found to have had 14 dead bird reports in the previous 7 days (June 7–14); only 5 were expected on the basis of the baseline period (April 1–May 24) (p=0.06) (Figure 2a). By June 17, all of Staten Island and an adjacent part of Brooklyn were included in the dead bird cluster (observed = 36, expected = 19, p=0.02). The first laboratory evidence of WNV from New York City was reported from Staten Island 1 month later, from two dead birds collected on July 5 and a mosquito pool collected on July 7 (Figure 2b). Spraying insecticide for adult mosquitoes was conducted on July 19. The first human case in 2000 was a resident of Staten Island whose illness began on July 20 and was diagnosed on July 28 (Table). During the next 2 months, Staten Island would prove to be the epicenter of the WNV encephalitis human outbreak in 2000, with most of the human cases and positive mosquito pools in New York City (Figures 2c and 2d). Dead bird clustering was also apparent in western Brooklyn (June 17), eastern Queens (July 6), and southern Brooklyn (August 16)—all sites that subsequently had numerous WNV-positive birds and mosquitoes and human cases (Figure 2 and Table). Only one patient's onset of illness was not preceded by dead bird clustering near his place of residence. That patient was a construction worker living in Manhattan, who attributed his illness to mosquito bites he sustained while working on an outdoor construction project in eastern Queens.

Year 2001 Implementation

In 2001, data for dead bird clustering analysis were first available on June 22. A retrospective analysis of dead bird

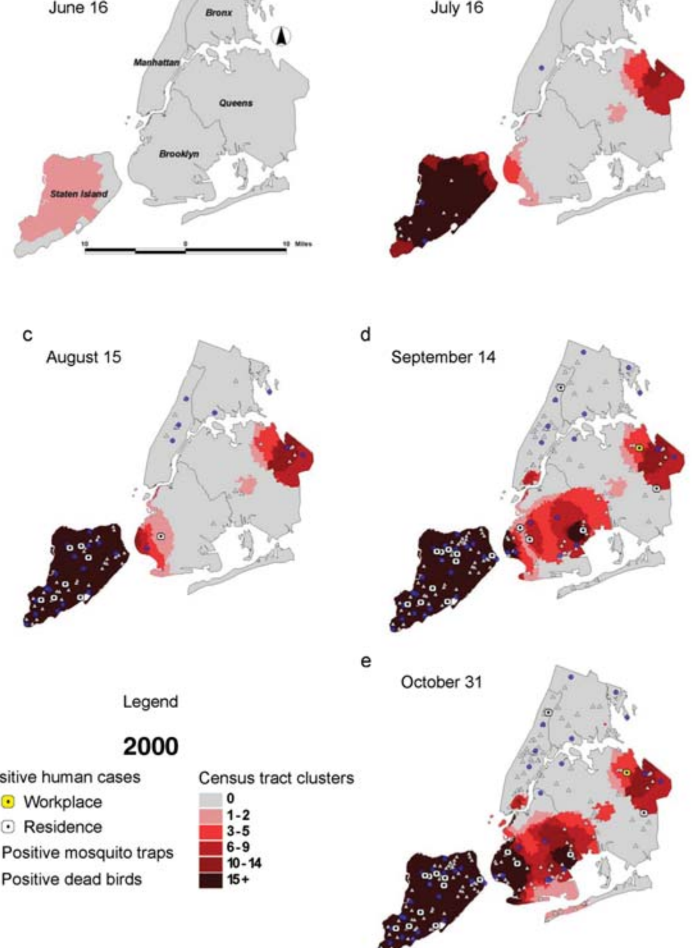

Figure 2. Dead bird clusters, West Nile virus (WNV)-positive dead birds, human cases, and mosquito traps, New York City, 2000. The shading represents the cumulative frequency of dead bird clusters in each census tract as of the date of analysis. Cumulative WNV-positive birds and mosquitoes are displayed on the basis of their date of collection; human cases are shown on the basis of their date of onset of illness.

reports before this date showed repeated clusters in central Staten Island (Figure 3a). Prospective real-time surveillance beginning June 22 found repeated clustering in eastern Queens, which prompted a program of intensified larval surveillance and control as well as abatement of standing water starting June 27. Reports from this area were prioritized for dead bird pickup and testing, and additional mosquito traps were set. On July 19, the clustering in eastern Queens was shown to be likely due to WNV through laboratory confirmation in a pool of mosquitoes collected on July 3 and a hatch-year live bird sampled on July 6 (Figure 3b). During the next 2 months, six areas with major dead bird clustering were identified, prompting intensified surveillance activities. Although active surveillance for human infections was implemented citywide, five of seven diagnosed human cases in 2001 were identified in residents of four cluster areas (Staten Island, eastern Queens, South Brooklyn, East Brooklyn). An additional human case occurred in a homeless person who frequented an area close to the southern Queens cluster area.

Dead bird reporting and analysis were interrupted by the September 11 attacks on the World Trade Centers. The subsequent low number of reports contributed to widespread geographic clusters, containing only a small number of dead bird reports and encompassing most of Brooklyn and Manhattan. The seventh diagnosed human case in the city was in a resident of a cluster area in lower Manhattan. In all, dead bird clusters occurred 0–40 days (median 12) before the onset of human illness and 12–45 days (median 17) before human diagnosis. In most cases, dead bird clusters also preceded time of collection of WNV-positive mosquitoes and birds (Table).

Discussion

As WNV continues its spread throughout the Western Hemisphere, jurisdictions will be looking for ways to perform surveillance for early virus activity. Forty-eight states and the District of Columbia have already established procedures for reporting, collecting, and testing dead birds (12). While mosquito trapping and testing remain the accepted standard of arboviral surveillance, use of routinely collected dead bird reports to detect WNV-related dead bird clusters may facilitate early detection and targeting of scarce surveillance and vector control resources.

The spatial scan statistic has proved useful for retrospective geographic disease surveillance for a variety of chronic diseases including breast cancer (13), Creutzfeldt-Jakob disease (14), and systemic sclerosis (15). In these applications of the spatial scan statistic, expected counts could be directly calculated by using the underlying population density or the geographic distribution of contemporaneous controls. However, data from some surveillance systems can manifest significant nonrandom geographic clustering at baseline because of variability in disease incidence, diagnosis, and reporting, all factors that are strongly affected by human behavior and not easily measured or controlled for in statistical analyses. The approach described here controls for the baseline spatial clustering in surveillance data, by searching instead for a change in the geographic distribution of recent events compared to an historical baseline. This approach implements for the first time such a spatial-temporal surveillance system in real time. This study may prove useful for early detection of other infectious disease outbreaks and for bioterrorism surveillance by using prediagnostic clinical or consumer

Table. Date of first West Nile virus (WNV) findings and response in communities with diagnosed human WNV infections[a]

Y	Date of first cluster	Positive bird report (collection date)	Positive mosquito collection	Human case report (onset date)
2000				
Staten Island	**6/14**	7/16 (7/5)	7/7	7/28 (7/20)
W. Brooklyn	**6/17**	8/15 (8/2)	8/24	8/24 (8/16)
E. Queens	**7/6**	7/20 (7/6)	7/23	9/21 (9/13)
S. Brooklyn	8/16	**8/14 (7/31)**	8/18	9/12 (8/27)
N Manhattan	None	8/2 (7/25)	**7/25**	10/17 (8/31)[b]
2001				
Staten Island	**5/25**[c] **7/2**	7/19 (7/5)	7/26	8/10 (7/26)
Staten Island	**5/25**[c] **7/2**		7/6	(8/5)
E. Queens	**7/5**	7/19 (7/6)	8/26	8/21 (8/14)
S. Queens	None	**8/16 (8/2)**	9/26	9/18 (9/7)[d]
E. Brooklyn	9/11	10/26 (9/6)	**9/6**	10/1 (9/9)
S. Brooklyn	**8/25**	8/31 (8/15)	9/11	9/11 (9/2)
Manhattan	9/23	-	**8/10**	10/11 (10/6)

[a]Dates in bold are the first surveillance data found in each area.
[b]Possible exposure in eastern Queens.
[c]Retrospectively determined.
[d]Exact residence not known.

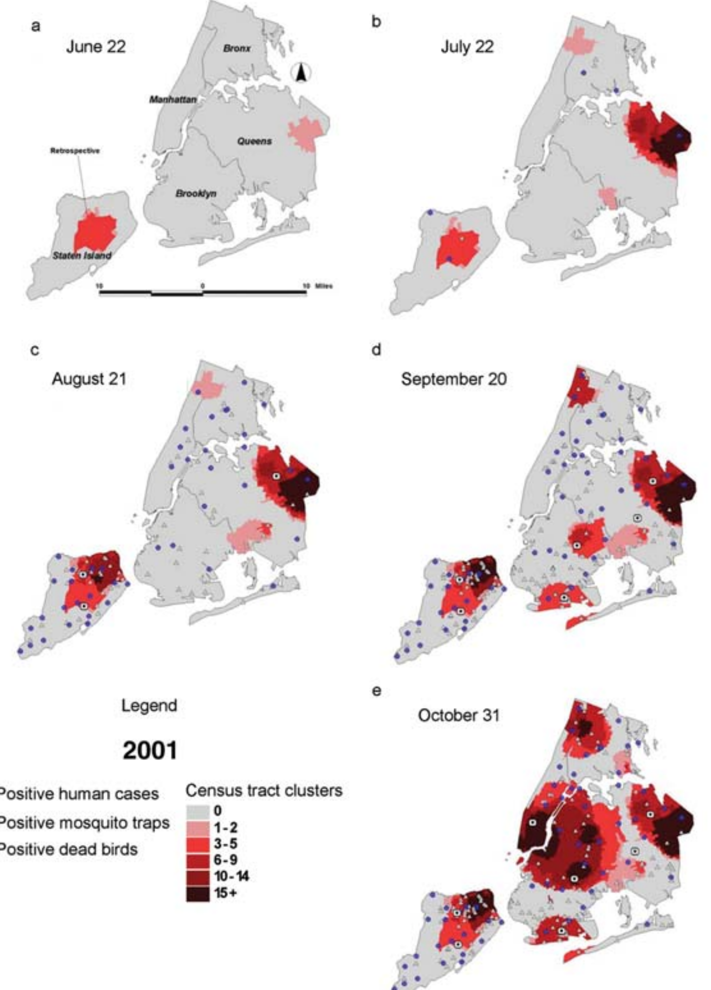

Figure 3. Dead bird clusters, West Nile virus (WNV)–positive dead birds, human cases, and mosquito traps, New York City, 2001. The shading represents the cumulative frequency of dead bird clusters in each census tract as of the date of analysis. Cumulative WNV-positive birds and mosquitoes are displayed on the basis of their date of collection; human cases are shown on the basis of their date of onset of illness.

data ("syndromic surveillance"). At the New York City Department of Health and Mental Hygiene, a syndromic surveillance system for the early detection of natural or bioterrorism-related outbreaks has been implemented using a similar prospective model.

In the current study, cluster analysis of routinely geocoded dead bird reports provided an early warning of small-area viral amplification in birds and mosquitoes and of subsequent human infections. Prospective implementation of this system in 2001 enabled preemptive measures to reduce mosquito breeding 4 weeks before WNV activity was laboratory confirmed in vertebrate hosts and arthropod vectors.

Where WNV infection is identified in mosquitoes or birds, dead bird clustering may provide additional confirmation of an ongoing epizootic as well as help define the geographic area of increased human risk. After laboratory evidence of WNV was found in dead birds in Staten Island on July 16, 2000, adult mosquito insecticide application was initially limited to a 2-mile radius around where the birds had been found. Dead bird clustering analysis would have provided early evidence of an intensifying epizootic throughout all of Staten Island. Further research is needed regarding use of flexible time windows and exact coordinates (rather than census tracts) in defining dead bird clusters. Also, the sensitivity and specificity of various criteria for defining an area of risk (e.g., statistical significance level, distance from dead bird cluster, persistence of clustering) must be further defined. We are currently exploring the use of various metrics, including Receiver Operating Characteristic (16) and Activity Monitor Operating Characteristic (17) curves, in evaluating these criteria.

The use of dead bird reporting has several limitations. First, dead birds may cluster in space and time for reasons other than WNV (e.g,. poisoning); intensified surveillance and investigation are needed to determine whether a cluster is due to the virus. Second, dead bird reporting is largely dependent on public sightings of dead birds and the public's interest in reporting them. While our clustering technique accounts for pre-outbreak baseline levels of dead bird reporting, any geographically localized media coverage can cause a clustering in dead bird reports; conversely,

areas with very low human populations, low interest in dead bird reporting, or other dead bird reporting mechanisms (e.g., parks) may have persistently low reports of dead bird despite a WNV epizootic. Finally, current avian deaths caused by WNV in North America may decrease over time because of natural selection for resistance to the virus among native bird species.

Through spatial-temporal cluster analysis of dead bird reporting data, jurisdictions can initiate early larval control activities, prioritize birds for testing, and triage scarce mosquito-collection and laboratory resources. All these activities enable more effective and efficient prevention of human disease caused by WNV. This adaptation of the spatial scan statistic for prospective outbreak detection could be useful in other infectious disease surveillance systems, including those for bioterrorism.

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Dr. Mostashari is an assistant commissioner at the New York City Department of Health. He is a medical epidemiologist with an expertise in nontraditional disease surveillance and outbreak detection. He was a lead investigator in the outbreaks of West Nile virus and anthrax in New York City.

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Gnathostomiasis: An Emerging Imported Disease

David A.J. Moore,* Janice McCroddan,† Paron Dekumyoy,‡ and Peter L. Chiodini†

As the scope of international travel expands, an increasing number of travelers are coming into contact with helminthic parasites rarely seen outside the tropics. As a result, the occurrence of *Gnathostoma spinigerum* infection leading to the clinical syndrome gnathostomiasis is increasing. In areas where *Gnathostoma* is not endemic, few clinicians are familiar with this disease. To highlight this underdiagnosed parasitic infection, we describe a case series of patients with gnathostomiasis who were treated during a 12-month period at the Hospital for Tropical Diseases, London.

The ease of international travel in the 21st century has resulted in persons from Europe and other western countries traveling to distant areas of the world and returning with an increasing array of parasitic infections rarely seen in more temperate zones. One example is infection with *Gnathostoma spinigerum*, which is acquired by eating uncooked food infected with the larval third stage of the helminth; such foods typically include fish, shrimp, crab, crayfish, frog, or chicken. Previously, most disease related to *Gnathostoma* was reported from Southeast Asia, particularly Thailand and Japan, because of the dietary habits of those living there. In recent years, however, gnathostomiasis has become an increasing problem in Central and South America, most notably in Mexico (perhaps related to consumption of ceviche) (1,2). In cats and dogs, which serve as important reservoirs of infection in regions where *Gnathostoma* is endemic (3), the ingested third-stage larva matures into the adult worm in approximately 6 months (Figure 1). However, because the larva cannot mature into the adult form in humans, the third-stage larva can only wander within the body of the host; clinical symptoms of gnathostomiasis then occur because of the inflammatory reaction provoked by these migrating larvae (Figure 2).

Traditionally the disease has been divided into cutaneous and visceral forms, depending on the site of larval migration and subsequent symptoms. Another form of gnathostomiasis, which is quite rare, includes the dangerous complication of central nervous system involvement (4). This form is manifested by painful radiculopathy, which can lead to paraplegia, sometimes following an acute (eosinophilic) meningitic illness.

We describe a series of patients in whom *G. spinigerum* infection was diagnosed at the Hospital for Tropical Diseases, London; they were treated over a 12-month period. Four illustrative case histories are described in detail. This case series represents a small proportion of gnathostomiasis patients receiving medical care in the United Kingdom, in whom this uncommon parasitic infection is mostly undiagnosed.

Methods

The case notes of patients in whom gnathostomiasis was diagnosed at the Hospital for Tropical Diseases were reviewed retrospectively for clinical symptoms and confir-

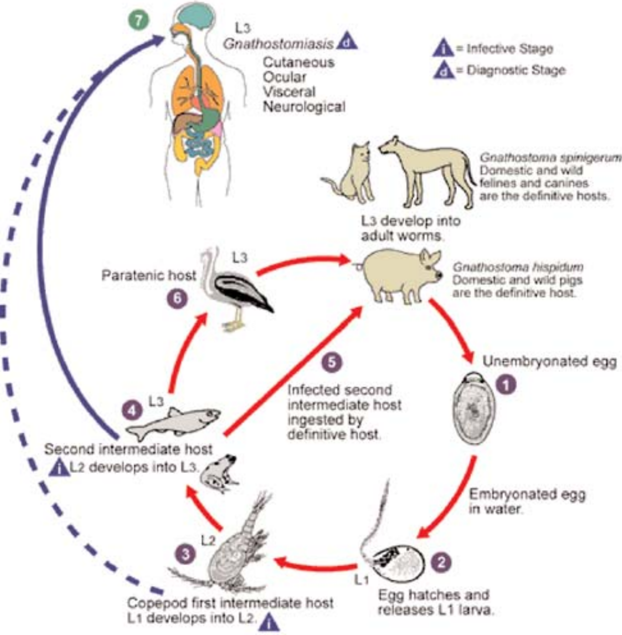

Figure 1. Life cycle of *Gnathostoma spinigerum*. Adapted from an original illustration by Sylvia Paz Diaz Camacho; available from: URL: http://www.dpd.cdc.gov/dpdx/ HTML/gnathostomiasis.htm

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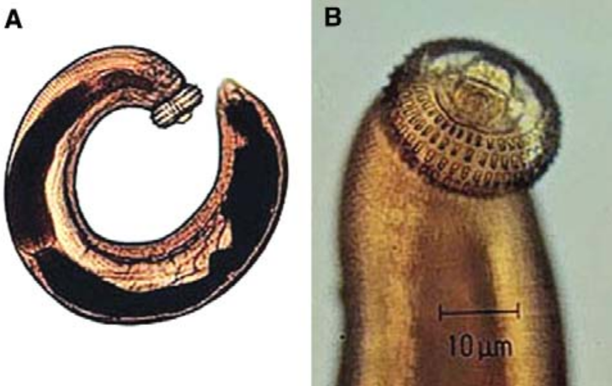

Figure 2. Third-stage larva of *Gnathostoma spinigerum*. A) whole larva; B) head. (Reproduced with the permission of Pichart Uparanukraw, Department of Parasitology, Faculty of Medicine, Chiang Mai University, Thailand.)

matory serologic results for the period April 1, 2000, to March 31, 2001. Clinical and laboratory data gleaned from case notes are described in the following sections.

Definitions

The definition of clinical *Gnathostoma* infection is: 1) a history of intermittent, migratory skin and subcutaneous swellings (localized or not localized) with or without peripheral blood eosinophilia (eosinophil count >0.4 x 10^9/L), or 2) otherwise undiagnosed eosinophilia with nonspecific symptoms. Plausible epidemiologic risk is defined as travel to an area in which gnathostomiasis had been reported previously (i.e., Southeast Asia and Central and South America). We did not impose a time limit on previous travel in our study. Positive *Gnathostoma* serologic results were defined as the presence on immunoblot of the specific 24-kDa band diagnostic of *Gnathostoma* infection (5,6). All serologic testing for gnathostomiasis was performed in the Department of Helminthology of the Faculty of Tropical Medicine at Mahidol University in Bangkok, Thailand. For patients at risk of *Loa loa* infection (because of previous travel to regions in central or West Africa where the infection is endemic), day-blood tests (samples taken between 12:00–2:00 p.m.) were performed to check for microfilaria and a filaria enzyme-linked immunosorbent assay was performed to exclude this diagnosis (Calabar swellings, indicative of *Loa loa* infection, may mimic gnathostomiasis).

Results

During the 12-month study period, we identified 16 patients who had clinical symptoms consistent with *Gnathostoma* infection, a plausible epidemiologic risk, and positive serologic results. Seven patients were referred by their general practitioner (primary care physician) and four by consultant physicians working elsewhere in London. Median time from onset of symptoms to diagnosis was 12 months (range 3 weeks–5 years). A dietary history was recorded for three patients who reported eating (among other things) raw fish and watercress (patient 1); mutton, fish, and chicken in Bangladesh (patient 3); and fish and a variety of crustacea from market stalls in Southeast Asia (patient 13). Eosinophilia was noted in seven patients and was usually modest, always declining after treatment. Median erythrocyte sedimentation rate (available for 12 patients, data not shown) was 10 (range 1–62). The countries visited most frequently by our 16 patients were India (n=4), Bangladesh (n=3), China (n=2), and Thailand (n=2). Standard treatment during the period of study was albendazole (400 mg twice a day for 21 days). Three patients required a second course for recurrence of symptoms and incomplete resolution of eosinophilia.

Case Histories

Detailed travel histories for these patients are described in the Table. The following sections include a case history for four patients; all of these patients had positive *Gnathostoma* serologic results and responded to albendazole therapy.

Case 1

A 26-year-old Asian woman, a resident of Hong Kong, was referred to our hospital by her primary care physician. She complained of the episodic appearance of “irritating” lumps on her limbs. Nine months earlier, the first of these lumps appeared on her right hand; since then, she had had a similar lump on her left foot and left hand, each lasting a few days and resolving spontaneously with no visible or palpable sequelae. Nine years previously, she had noted a lump rising near her left knee, which was followed 4 days later by a similar lump on her right thigh; both lumps had resolved spontaneously. All of the lumps were subcutaneous and estimated at 3–6 cm in diameter. A positive rheumatoid factor and anti-nuclear antibody >1:1,280 were noted. Her diet frequently included raw fish.

Case 2

A 37-year-old woman from Bangladesh reported a 3-year history of intermittent swelling of the right forearm and upper arm to the midbiceps area associated with pruritus, myalgia, and arthralgia. The onset of her symptoms had occurred while she was visiting Bangladesh, where she had eaten mutton, fish, and chicken. An eosinophil count of 4.37 x 10^9/L had prompted referral from a rheumatology clinic to the Hospital for Tropical Diseases. After a 21-day course of albendazole, her eosinophil count decreased to 1.12 x 10^9/L; symptoms recurred several months later. After treatment with a second course of albendazole (400 mg twice a day for 21 days), her

Table. Background information on patients in whom *Gnathostoma* infection was identified, April 1, 2000, to March 31, 2001, Hospital for Tropical Diseases, London[a]

Patient no.	Age	Referral source	Travel history	Eosinophil count ($x\ 10^9/L$)[b]	Symptom duration
1[c]	26	GP	China, South Korea, Canada, Hong Kong, Tunisia	0.10	9 mo
2	26	General physician	Bangladesh, Italy	2.20	6 mo
3[c]	37	Rheumatologist	Bangladesh	4.37	3 y
4	28	HTD walk-in	Japan, Cuba	0.17	2 mo
5	35	GP	India, Sri Lanka	NA	3 y
6	34	HTD walk-in	South Africa, New Zealand, Jakarta, Singapore	0.80	3 mo
7	49	Dermatologist	India, Thailand	0.1	13 mo
8	51	GP	Sri Lanka, Brazil, Cambodia	0.08	2 y
9	26	Rheumatologist	India	1.33	3 y
10	27	GP	Bangladesh	1.10	5 y
11	23	GP	SE Asia, Australia	0.00	4 mo
12	25	self	Japan, SE Asia, USA, Canada	0.11	13 mo
13	24	HTD walk-in	SE Asia, India, China	0.96	3 wk
14[c]	49	Gastroenterologist	Far East, Caribbean, USA	0.95	12 mo
15	57	GP	Vietnam, Thailand	0.26	6 mo
16[c]	30	GP	Borneo, Belize, Ecuador, Peru, Australia	0.11	12 mo

[a]GP, general practitioner; HTD walk-in, Hospital for Tropical Diseases emergency walk-in clinic; NA, not available.
[b]Normal range 0–0.4 x 10^9/l.
[c]Denotes case history in text.

symptoms resolved and her eosinophil count returned to normal (0.25 x 10^9/L).

Case 3

A 49-year-old Caucasian woman complained of a 12-month history of abdominal pain and symptoms suggesting gastroesophageal reflux. She had traveled widely in Southeast Asia 18 months earlier but denied eating crustacea or non-kosher meat. Gastric biopsy at upper gastrointestinal endoscopy demonstrated eosinophilic gastritis (peripheral blood eosinophil count of 0.95 x 10^9/L), a finding that prompted serologic testing for *Gnathostoma*. Her symptoms resolved with albendazole treatment.

Case 4

Pain developed in the left thigh of a 30-year-old man while he was participating in the Eco-Challenge 2000 race in Borneo. A 4x3-cm lump in his thigh was initially attributed to a muscular tear; when this lump persisted for 12 months, he was referred to the Hospital for Tropical Diseases. Magnetic resonance imaging of the thigh (Figure 3) showed a lobulated lesion in the vastus lateralis muscle surrounded by edema. Serologic results were positive for *Gnathostoma*. Treatment with albendazole substantially reduced the size and firmness of the lesion but did not completely resolve it.

Discussion

This series is the first reported set of travelers with gnathostomiasis. Patterns of international travel suggest that this condition may be seen more often in travelers and immigrants from regions in which the disease is endemic. Moreover, the widening geographic distribution of the infection and increasingly adventurous eating habits of visitors to such regions are likely to contribute to an increase in incidence.

In our patients, the median time from onset of symptoms to diagnosis was 12 months, which reflects both the intermittent, episodic nature of the symptoms and the

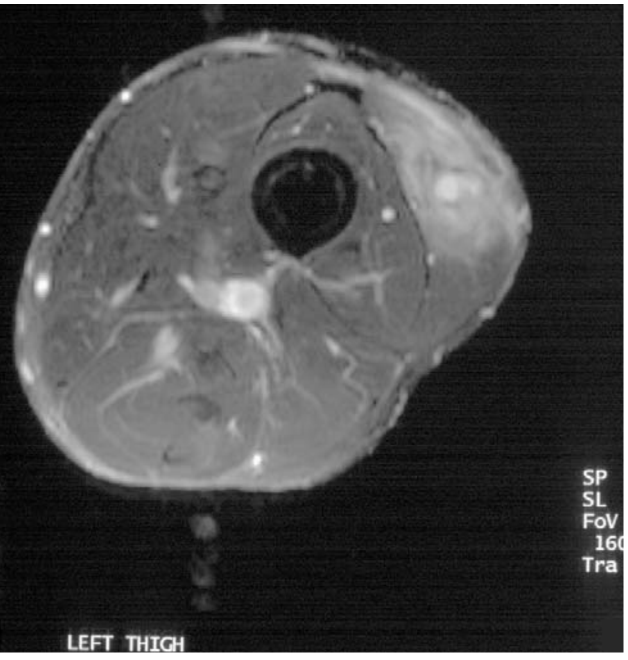

Figure 3. Magnetic resonance image of thigh with *Gnathostoma* larva (case 4).

obscurity of the diagnosis. We do not have data on the time to diagnosis after medical attention was sought, but anecdotally we often and understandably find a considerable delay.

The key to diagnosis of gnathostomiasis is recognition of the highly suggestive clinical history; cases 1 and 2 are the most typical. Once the disease is diagnosed, management is straightforward, but the rarity of the condition in areas in which the condition is not endemic might lead to the diagnosis' being overlooked. The unusual symptoms, combined with the usual absence of physical signs between episodes, may lead to discounting of the symptoms and erroneous reassurance of the patient by clinicians unfamiliar with gnathostomiasis. Patients may be referred to rheumatology, dermatology, or general medical clinics; the absence of eosinophilia may also prevent due consideration of possible parasitic causes. Eosinophilia was present in only seven of our patients and thus cannot be considered as a screening tool. However, as a marker of treatment response in those with eosinophilia at baseline, this investigation was proven useful; for the three patients requiring a second course of albendazole, residual eosinophilia preceded symptom relapse.

Because of little information about dietary intake, we cannot comment on the sources of infection in our patients. More detailed dietary histories are now recorded routinely at the Hospital for Tropical Diseases, but the notorious inaccuracy of verbal dietary histories and the broad range of potential culprits eaten by many travelers suggest that, for identifying the source in humans, dietary history is usually of limited value.

A number of serologic tests are available for the diagnosis of gnathostomiasis. Our testing is performed at Mahidol University in Thailand by using an immunoblot to detect the specific 24-kDa band considered diagnostic of *Gnathostoma* infection. In that laboratory, for the four parasite-confirmed cases of *Gnathostoma*, the immunoblot was 100% sensitive, and antibodies of 15 parasitic diseases and one mixed infection were not cross-reactive, except for 1 of 13 samples from patients with paragonimiasis which gave a weak reaction against this antigen (5). Antibodies from 16 patients with confirmed cases of *Gnathostoma* were consistently reactive with this 24-kDa antigen. Cross-reactivity was not found in a further extensive study of parasitic and nonparasitic diseases (6).

The reported efficacy of albendazole in the treatment of gnathostomiasis is >90% (7,8), and similar success has been reported for ivermectin (8). Three of our patients required a second course of treatment. The episodic nature of this condition means that an initial determination is difficult as to whether cure has been effected, but the resolution of eosinophilia and lack of symptom recurrence within 12 months were taken as presumptive evidence of cure. Although we used a second course of albendazole for retreatment, ivermectin has also been used successfully (9).

A diagnosis of gnathostomiasis should be considered for patients with a history of transient, migratory cutaneous or subcutaneous swellings, or nonspecific gastrointestinal symptoms for which a potential epidemiologic exposure is identified. Management of the disease thereafter is usually relatively straightforward, although more than one course of treatment may be required to effect a cure.

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Histopathologic Features of *Mycobacterium ulcerans* Infection

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Because of the emergence of Buruli ulcer disease, the World Health Organization launched a Global Buruli Ulcer Initiative in 1998. This indolent skin infection is caused by *Mycobacterium ulcerans*. During a study of risk factors for the disease in Ghana, adequate excisional skin-biopsy specimens were obtained from 124 clinically suspicious lesions. Buruli ulcer disease was diagnosed in 78 lesions since acid-fast bacilli (AFB) were found by histopathologic examination. Lesions with other diagnoses included filariasis (3 cases), zygomycosis (2 cases), ulcerative squamous cell carcinomas (2 cases), keratin cyst (1 case), and lymph node (1 case). Thirty-seven specimens that did not show AFB were considered suspected Buruli ulcer disease cases. Necrosis of subcutaneous tissues and dermal collagen were found more frequently in AFB-positive specimens compared with specimens from suspected case-patients ($p<0.001$). Defining histologic criteria for a diagnosis of Buruli ulcer disease is of clinical and public health importance since it would allow earlier treatment, leading to less deforming sequelae.

Mycobacterium ulcerans produces an indolent cutaneous infection known as Buruli ulcer disease (1–4). Three clinical stages of lesions have been described: preulcerative (which can present as a nodule, papule, plaque, or edema), ulcerative, and healed (scar) disease (5). The clinical characteristics of these lesions are nonspecific, particularly during the preulcerative stage. Lesions usually start as a single, painless, subcutaneous nodule, ill-defined edema, or plaque that enlarges over time. The skin that covers the nodule or plaque eventually sloughs off, together with the underlying tissues, forming an ulcer. If left untreated, the ulcer enlarges and becomes undermined. The patient usually has no systemic symptoms. Spontaneous healing of the ulcer has been described; healing starts at the proximal end of the ulcer and extends to the distal portions, resulting in a depressed scar that contracts and may produce severe deformities (1).

Many diseases can be confused with Buruli ulcer disease in each of its clinical stages; thus, laboratory tests and procedures can help establish the diagnosis (4). Such methods include culturing for *M. ulcerans* from lesion samples, testing swab samples of ulcers for acid-fast bacilli (AFB), histopathologic screening for characteristics of the disease, using polymerase chain reaction (PCR) to find bacterial DNA; or all of the above (3–7). Of the previously enumerated methods, only culture can be considered specific for detection of *M. ulcerans,* but it has a low sensitivity (3–5). Several authors have described the histopathologic changes of Buruli ulcer disease as the patients progress through the different clinical stages (8,9). However, letting a patient progress to a diagnostic ulcer is unacceptable. Thus, better defining diagnostic histopathologic features, particularly in the early stages, is of clinical and public health importance since this will allow early treatment and lead to less deforming sequelae.

M. ulcerans has been identified in many tropical and temperate parts of the world, and in the last decade reports of the disease have increased in several West African countries, including Ghana (3,10). As part of a study of surveillance, serodiagnosis, and identification of risk factors for Buruli ulcer disease in Ghana, excisional skin-biopsy specimens were obtained from clinically suspect lesions. We review the histopathologic examination of the skin specimens from the patients from Ghana and list the differential diagnoses encountered in preulcerative and ulcerative lesions. We compare the histopathologic features of AFB-positive and AFB-negative specimens and correlate pathologic examination, PCR, and culture results.

Materials and Methods

A total of 144 excisional skin-biopsy specimens were obtained from patients with clinically suspect lesions at therapeutic centers of excellence for Buruli ulcer disease in three highly disease-endemic districts in Ghana. Specimens were fixed in formalin and transported to the Infectious Disease Pathology Activity at the Centers for

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Disease Control and Prevention, Atlanta, GA. Representative portions of the specimens, measuring about 2.5 x 1.5 x 0.5 cm, were embedded in paraffin in one cassette, and sections were stained with hematoxylin and eosin (H&E) and Ziehl-Neelsen (to highlight AFB). Table 1 describes the histopathologic features evaluated. One pathologist (JG) searched for AFB, using the 40X magnification objective through the entire section (each slide was reviewed for 45 to 60 min). When a section did not have AFB bacilli, additional tissue specimens with approximately the same dimensions as the first were embedded in paraffin and studied with H&E and Ziehl-Neelsen stains. In AFB-negative cases, fungal causes of nodules and ulcers were searched by using Grocott methenamine silver.

Cases in which the specimen lacked subcutaneous adipose tissue were excluded from analysis. Specimens in which AFB were found in histologic sections were considered definite cases; specimens with negative AFB were considered suspected cases unless they had other diagnoses that could account for a clinical nodule or ulcer. Confirmation of histopathologic diagnosis of Buruli ulcer disease was possible in 62 cases by using culture, PCR for *M. ulcerans,* or both. Culture and PCR (IS2404) were performed according to standard techniques (4,11). Complete details of these techniques and a comparison of diagnostic methods will be published separately.

The frequency of diagnosis of nodules versus diagnosis of ulcers was determined. The histopathologic features of AFB-positive specimens compared to such features in AFB-negative specimens, and definite Buruli ulcer disease preulcerative lesions compared to ulcerative lesions were derived with SAS 8.2 (SAS Institute, Inc., Cary, NC) by using the chi-square and Fisher exact tests with a statistical significance of p=0.05. The same statistical tools were used to analyze frequencies of the histopathologic features in AFB-negative versus confirmed cases.

Table 1. Histopathologic features evaluated in definitive and suspected Buruli ulcer cases

Location, feature	Comments
Epidermis	
Hyperplasia	Psoriasiform (regular downward elongation of rete ridges), or pseudoepitheliomatous (irregular elongation of rete ridges)
AFB[a]	Presence or absence
Dermis	
Elastolysis	Collagen degeneration and necrosis seen as granular blue/purple collagen bundles with H&E stain
Inflammation, type	Acute (presence of neutrophils), chronic (presence of lymphocytes and macrophages), or granulomatous (presence of multinucleated giant cells and epithelioid histiocytes)
AFB	Presence or absence
Vascular changes	Thickening of the media, necrosis, and inflammation of vascular walls
Subcutis	
Necrosis	Coagulative or fat necrosis
Inflammation, type	Acute (presence of neutrophils), chronic (presence of lymphocytes and macrophages), or granulomatous (presence of multinucleated giant cells and epithelioid histiocytes)
Inflammation, intensity	Absent, mild (scattered inflammatory cells), or intense (inflammation forming nodules or bands)
AFB	Absent, mild (1–5 AFB seen with 40X objective), moderate ($\geq$6 AFB seen with 40X objective), or marked (AFB seen with 20X objective as clumps or colonies)

[a]H&E, hematoxylin and eosin stain; AFB, acid-fast bacilli.

Results

Adequate material for histopathologic review was available for 124 of 144 cases. Twenty cases (14%) were excluded because of lack of subcutaneous tissue in the biopsy specimen. The results of analysis of these 124 specimens are shown in Table 2. In summary, using histopathologic methods, we evaluated 30 (24%) nodules, 6 (5%) plaques, and 88 (71%) ulcers. By histopathologic examination, diagnoses other than Buruli ulcer disease were found for nine patients, seven of whom had nodules and two of whom had ulcers. Patients with nodules included three with parasites (two *Onchocerca volvulus* and one *Mansonella streptocerca*), two with deep fungi (subcutaneous zygomycosis), one with a keratin cyst, and one with a hyperplastic lymph node; the two patients with ulcers had squamous cell carcinoma. AFB were present in 78 (63%) specimens, while 37 (30%) specimens did not have AFB and were considered suspected cases of Buruli ulcer disease. Of 78 cases with positive AFB, bacilli were found in 69 (88%) of the first specimens submitted for pathology and 9 (11%) when additional tissue (available in 48 of the original AFB-negative specimens) was studied. We had a histopathologic diagnosis in 25 (83%) of the 30 nodules, whether this was definite Buruli ulcer disease or another diagnosis; the proportion of pathology diagnosis was lower for ulcers (57 [65%] of 88). The proportion of nodules and ulcers with a definite diagnosis of Buruli ulcer disease was approximately the same (60% vs. 62%).

The histopathologic features for AFB-positive and AFB-negative cases are shown in Table 3. Necrosis of the subcutaneous tissues was found in 100% of AFB-positive and 62% of AFB-negative cases (p<0.001). Sixty-one percent of AFB-positive cases and 6% of AFB-negative cases had necrotic collagen in the dermis (elastolysis) (p<0.001). In 92% of AFB-positive cases, the inflammatory infiltrate had neutrophils mixed with mononuclear cells; by contrast, suspected cases had a predominance of mononuclear inflammation (p=0.008). Epidermal hyperplasia (either psoriasiform or pseudoepitheliomatous), chronic and granulomatous inflammation, and vasculopathy were found at

Table 2. Number (percent) of specimens with other diagnoses, definite, and suspected Buruli ulcer according to clinical stage[a]

Clinical stage	Other diagnoses	Definite BU (AFB positive)	Suspect BU (AFB negative)	Total
Nodule	7 (6)	18 (14)	5 (4)	30 (24)
Plaque	0	5 (4)	1 (0.8)	6 (5)
Ulcer	2 (1.6)	55 (44)	31 (25)	88 (71)
Total	9 (7)	78 (63)	37 (30)	124 (100)

[a]BU, Buruli ulcer; AFB, acid-fast bacilli.

approximately the same rate for both AFB-positive and -negative cases. Duration of the lesion was available for 113 cases; 70 were positive for AFB, 35 were negative, and 8 had other diagnoses. Forty-seven (67%) AFB-positive cases had lesions that had been present for ≤3 months, with a median of 2 months (range 0.2–36); for AFB-negative cases, 19 (54%) had lesions that were present ≤3months, with a median of 3 months (range 0.2–156) (p=0.64).

The frequency of the histopathologic variables for preulcerative and ulcerative stage of AFB-positive cases is shown in Table 4. Variables that showed significant association with the ulcerative stage included epidermal hyperplasia (p=0.005), intense chronic inflammation (p=0.013), and granulomas (p=0.005). Dermal elastolysis was more frequent in preulcerative lesions (p=0.015). Of note is the lack of a statistically significant difference between preulcerative and ulcerative lesions for the concentration of AFB in the subcutaneous tissues (p=0.07). Psoriasiform epidermal hyperplasia was found in 47 Buruli ulcer disease cases, 7 in preulcerative lesions and 40 in ulcers. Pseudoepitheliomatous hyperplasia was found in 27 cases, 3 with preulcerative lesions and 24 with ulcers. AFB in the keratin were found in one nodule and in seven ulcer cases. Figure 1 shows a photomicrograph of a nodule, and Figure 2 shows an ulcer from a definitive case.

Of the 37 AFB-negative cases, 1 had a positive culture, and 22 had *M. ulcerans* nucleic acids detected by PCR. However, positive PCR results were also obtained from samples of cases with filarial nodules (three patients), keratin cyst, deep fungi, and squamous cell carcinoma (one patient each). Analysis of hsitopathologic features showed a significant association of necrosis of subcutaneous tissues and elastolysis (p=0.0009 and p<0.0001, respectively) with confirmed cases compared to AFB-, culture-, and PCR-negative cases.

Discussion

Necrosis of subcutaneous tissues and dermal collagen accompanied by minimal inflammation and AFB are considered the most reliable histopathologic features for the diagnosis of Buruli ulcer disease (8,9,12). Our study demonstrated that necrosis of both the subcutis and dermal collagen was significantly associated with cases. The necrosis found in such cases has been attributed to a polyketide (called mycolactone) that is produced by *M. ulcerans* and acts as an extracellular toxin (13–16). AFB-positive cases showed a significant association with the presence of neutrophils mixed in the necrotic material. In Buruli ulcer disease cases, inflammation appears to be minor for the amount of necrosis, which accounts for previous descriptions of minimal inflammatory response. Possibly, the toxin that induces adipose cell necrosis also induces necrosis of the inflammatory infiltrate.

Several authors have established that during the preulcerative stage and early in the ulcerative stage, the coagulative necrosis forms a nidus where calcifications and AFB colonies are easily visualized (8,9,12,17). However, when the ulcer starts healing, and granulation tissue, fibrosis,

Table 3. Comparison of histopathologic features of definite and suspected Buruli ulcer cases

Histopathologic feature	Buruli ulcer (AFB positive)[a] no. (%)	Suspected Buruli ulcer (AFB negative)[a] no. (%)	p value
Epidermis[b]			
Hyperplasia	50/73 (68)	21/35 (60)	0.38
Dermis[b]			
Elastolysis	45/74 (61)	2/35 (6)	<0.0001
Subcutaneous tissue			
Necrosis	78/78 (100)	23/37 (62)	<0.0001
Vasculopathy	58/78 (74)	27/37 (73)	0.87
Acute inflammation[c]	72/78 (92)	27/37 (73)	0.008
Chronic inflammation[d]	31/78 (40)	18/37 (49)	0.36
Granulomas[e]	30/78 (38)	12/37 (32)	0.53

[a]AFB, acid-fast bacilli.
[b]Seven specimens did not have epidermis, and six did not have dermis.
[c]Acute inflammation considered as present versus absent.
[d]Chronic inflammation considered as intense versus mild.
[e]Granulomas considered as present versus absent.

Table 4. Comparison of histopathologic features of preulcerative and ulcerative lesions in definite Buruli ulcer cases

Histopathologic feature	Preulcerative no. (%)	Ulcerative no. (%)	p value
Epidermis			
Hyperplasia[a]	8 (42)	42 (78)	0.005
Dermis			
Elastolysis[a]	17 (85)	28(52)	0.015
AFB in dermis[b]	8 (40)	10 (19)	0.34
Subcutaneous tissue			
AFB in subcutis[c]	18 (78)	31 (56)	0.074
Acute inflammation[d]	22 (96)	50 (91)	0.48
Chronic inflammation[e]	4 (17)	27 (49)	0.013
Granulomas[f]	3 (13)	27 (49)	0.005

[a]Two specimens did not have epidermis, and one did not have dermis.
[b]Considered as presence of acid-fast bacili (AFB) in dermis.
[c]Considered as intense versus mild AFB in subcutis.
[d]Acute inflammation considered as present versus absent.
[e]Chronic inflammation considered as intense versus mild.
[f]Granulomas considered as present versus absent.

and granulomatous inflammation are present, AFB are difficult to document (8,9,17). Our study showed AFB in the keratin layer in seven of the Buruli ulcer disease ulcer cases and only in one nodule. AFB in keratin have been observed previously and may represent bacilli from colonies that are actively being sloughed off or they may be carry over from histologic processing (8). Contrary to findings in published reports, our study did not show a statistically significant difference in the amount of AFB in the subcutaneous tissues in the preulcerative and ulcerative stages. Additionally, the presence of AFB in clinically suspect lesions was not related to the duration of the lesion. These findings can be explained by any or all of the following factors: our study had a small number of cases in the preulcerative stage; our sampling techniques were geared to maximizing the amounts of AFB in the tissues (AFB have been observed more frequently in the distal portion of the ulcer); we obtained lesion samples at an earlier stage than other researchers. In addition, previous studies have not used statistical methods to analyze the frequency of histopathologic features; thus, the interpretation of results has been subjective.

Our study showed that epidermal hyperplasia and chronic inflammation with formation of granulomas were statistically more frequent in Buruli ulcers than in preulcerative lesions. All these features have been described to be more prominent in the later stages of disease (8,9). Epidermal regeneration occurs in an effort to cover the epidermal tissue defect (18). In our cases, the most frequent type of regeneration was psoriasiform, with some case-patients exhibiting pseudoepitheliomatous hyperplasia. Granulomatous inflammation is characteristic of persistent infections that evoke delayed hypersensitivity, as seen in several mycobacterial and fungal infections. Some authors have suggested that leprosy and Buruli ulcer disease may have similar gradation of the inflammatory process from foamy macrophages (lepromatous) to well-formed granulomas (tuberculoid) (9,19).

Currently, confirmation of clinically suspected cases of Buruli ulcer disease is based on culture of *M. ulcerans* from the tissues, presence of AFB in swab samples, evidence of *M. ulcerans* DNA, or characteristic histopathologic changes in tissue sections (3,4). Several problems are evident from this manner of confirming a diagnosis. The isolation rate of *M. ulcerans* from patients approaches only 35% because the bacteria are very difficult to culture (20). In our series, only one suspected case was confirmed by using culture. AFB in swab samples are rare and depend on the clinical stage and tissue sampled (3,4,8,20). None of the cases in this cohort was confirmed by AFB in swab

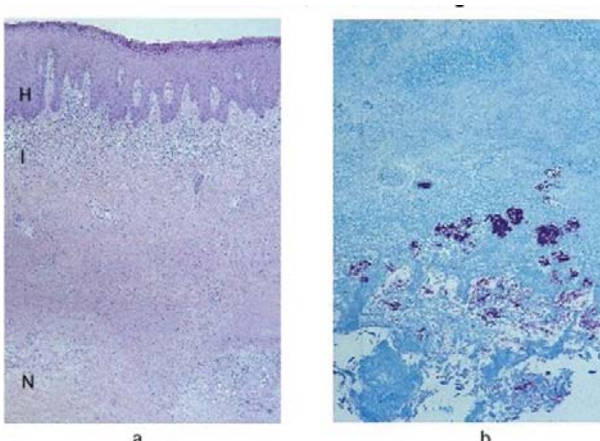
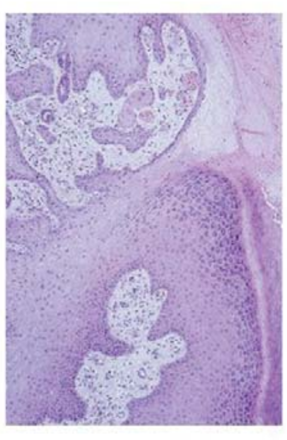
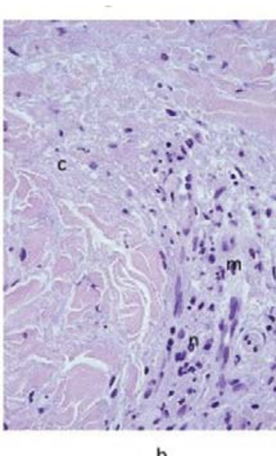


Figure 1. a, Hematoxylin and eosin stain of a lesion specimen showing definitive Buruli ulcer disease in the preulcerative stage (original magnification 50x). Notice the psoriasiform epidermal hyperplasia (H), superficial dermal lichenoid inflammatory infiltrate (I), and necrosis of subcutaneous tissues (N). b, Ziehl-Neelsen stain of the same nodule, showing abundant colonies of acid-fast bacilli in the necrotic subcutaneous tissues (original magnification 100x).





a b

Figure 2: a, Hematoxylin and eosin stain of the pseudoepitheliomatous hyperplasia of the epidermis in a lesion specimen showing definitive Buruli ulcer disease in the ulcerative stage (original magnification 100x). b, hematoxylin and eosin stain of the necrotic collagen (c) accompanied by mild inflammatory infiltrate in the dermis of a definitive Buruli ulcer disease lesion in the ulcerative stage (original magnification 400x). n, neutrophils; m, mononuclear cells.

specimens. PCR would conceivably be helpful, but no data are available on sensitivity and specificity with large numbers of clinical specimens (5,11). In this cohort, PCR showed positive results in cases with filarial nodules and a keratin cyst; since these patients had single nodules and did not have histopathologic evidence of Buruli ulcer disease, the findings probably represent false-positive PCR results. The cases with positive PCR results and squamous cell carcinoma and deep fungi could potentially have either two concomitant infections or a cancer arising from long-standing Buruli ulcer disease (21). Issues of DNA contamination, required technical expertise, and PCR costs prohibit this assay's utility as a routine clinical diagnostic tool in the field (3,5,13,14).

"Characteristic" histopathologic changes are considered one of the confirmatory laboratory methods for Buruli ulcer disease; however, the features are nonspecific and change as the lesion evolves from a nodule to an ulcer. In this study, definite histopathologic diagnosis of Buruli ulcer disease was only possible in 63% of cases because the presence of AFB bacilli was not always detectable even though necrosis of subcutaneous tissue and collagen were observed. An additional challenge in the histopathologic diagnosis of this disease is having adequate tissue samples. In our study, 14% of the specimens were considered inadequate because they lacked subcutaneous tissue, and among those with adequate material, 11% required additional tissue to demonstrate AFB. New techniques that can be applied to tissue are greatly needed to diagnose Buruli ulcer disease in all stages. Until these techniques are available, defining diagnostic histopathologic features of the disease will enable better understanding of clinicopathologic and pathogenetic characteristics of *M. ulcerans* infection.

For this study, we collected samples from more ulcers than nodules; however, a higher proportion of nodule specimens received a histopathologic diagnosis. The histopathologic differential diagnosis for ulcers included squamous cell carcinomas only; among the other clinical diagnoses that can be encountered in this stage are tropical phagedemic ulcer, actinomycosis, noma, leishmaniasis, yaws, and scrofuloderma (4). In our cohort, the histopathologic differential diagnosis for nodules was more extensive and included other infectious diseases (filaria and zygomycosis) as well as other noninfectious causes of skin nodules (keratin cyst) (4).

In summary, our study shows the histopathologic features of patients with clinically suspected Buruli ulcer disease in a population with a high prevalence of the disease. We found that necrosis of subcutaneous tissues and dermal collagen was the best predictor of disease; however, the histopathologic changes are not unique, and diagnosis requires correlation with the clinical picture and other laboratory techniques.

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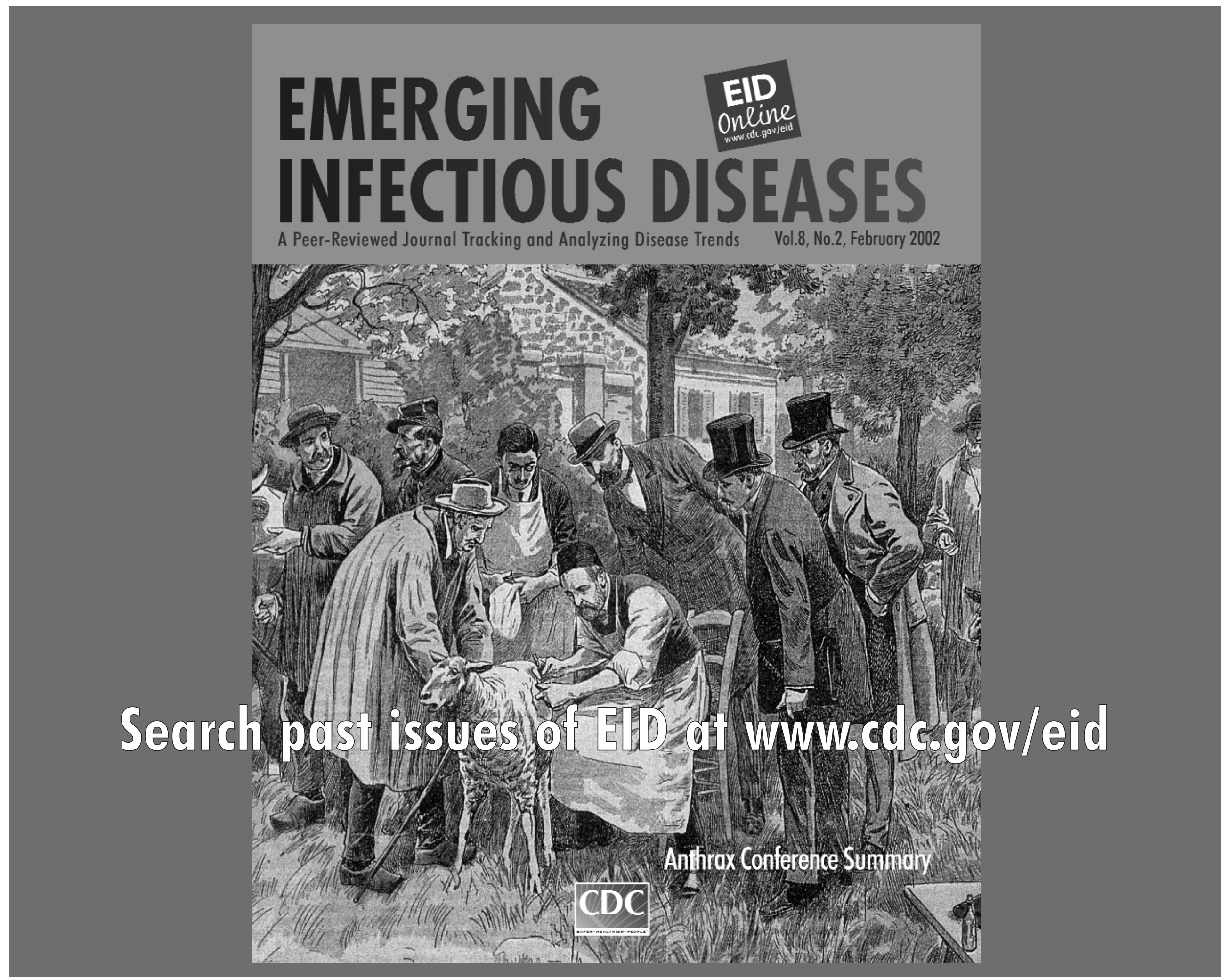

Clinical Implications of Varying Degrees of Vancomycin Susceptibility in Methicillin-Resistant *Staphylococcus aureus* Bacteremia[1]

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We conducted a retrospective study of the clinical aspects of bacteremia caused by methicillin-resistant *Staphylococcus aureus* (MRSA) with heterogeneously reduced susceptibility to vancomycin. Bloodstream MRSA isolates were screened for reduced susceptibility by using brain-heart infusion agar, including 4 mg/L vancomycin with and without 4% NaCl. Patients whose isolates exhibited growth (case-patients) were compared with those whose isolates did not (controls) for demographics, coexisting chronic conditions, hospital events, antibiotic exposures, and outcomes. Sixty-one (41%) of 149 isolates exhibited growth. Subclones from 46 (75%) of these had a higher MIC of vancomycin than did their parent isolates. No isolates met criteria for vancomycin heteroresistance. No differences in potential predictors or in outcomes were found between case-patients and controls. These data show that patients with vancomycin-susceptible MRSA bacteremia have similar baseline clinical features and outcomes whether or not their bacterial isolates exhibit growth on screening media containing vancomycin.

Staphylococcus aureus is an important cause of illness and death and accounts for about one-fifth of bacteremia cases in the United States (1). In 1997, Hiramatsu et al. reported the first clinical strain of methicillin-resistant *S. aureus* (MRSA) that exhibited reduced susceptibility to vancomycin (2). A report of other such isolates, classified as vancomycin-intermediate *S. aureus* (VISA), soon followed (1). Infection with VISA has been associated with vancomycin treatment failures, but it is a rare phenomenon, with worldwide prevalence limited to isolated case reports and a single limited outbreak (1,3). Rarer still in *S. aureus* is the phenomenon of vancomycin resistance (VRSA), with only two clinical VRSA isolates reported to date, both in 2002 (4,5).

Far more common than VRSA and VISA, however, are MRSA isolates that exhibit "heteroresistance" to vancomycin (hetero-VISA), whereby subpopulations within the strain exhibit reduced susceptibility, although the overall MIC for the isolate is within the susceptible range ($\leq$4 mg/L). A recent survey in Japan found this phenotype in up to 26% of clinical MRSA isolates collected in university hospitals (6).

Clinical laboratories do not perform heteroresistance testing for a number of reasons: Such testing is cumbersome, no standardized testing methods exist, and, perhaps most important, the clinical significance of this phenotype is not known (7). Although clinical MRSA isolates exhibiting hetero-VISA have now been reported from several countries (7–16), no study has demonstrated that patients with infections caused by these strains fare differently than patients with comparable infections caused by MRSA strains that are homogeneously susceptible to vancomycin.

Throughout this article, we use the following terms to describe different phenotypic features of *S. aureus* isolates: Vancomycin-susceptible *S. aureus* (VSSA) refers to isolates that are susceptible to vancomycin, according to NCCLS criteria (MIC $\leq$4 mg/L) (1). VISA refers to isolates that have intermediate susceptibility to vancomycin per NCCLS criteria (MIC 8–16 mg/L) (17). Hetero-VISA refers to isolates for which the MIC of vancomycin for one or more subpopulations is >4 mg/L, whereas the overall

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MIC is ≤4 mg/L. VRSA refers to isolates with an MIC of vancomycin ≥32 mg/L (17).

Many investigators who have looked at hetero-VISA (so named because the resistant subclones have intermediate susceptibility) have first screened for reduced susceptibility to vancomycin and then confirmed hetero-VISA status by demonstrating MICs above the susceptible range (i.e., >4 mg/L) among subclones of those isolates with positive screening results (6,9,10,12–16). No study has examined isolates that meet screening criteria yet fail to qualify as hetero-VISA on confirmatory testing. Such isolates are composed of subpopulations that, although susceptible to vancomycin, demonstrate varying degrees of susceptibility. Thus they may be capable of a certain degree of growth on screening media containing vancomycin, despite an absence of subpopulations that demonstrate intermediate resistance to vancomycin by MIC criteria. If, as has been suggested, VISA arise from homogeneously vancomycin-susceptible *S. aureus* through a multistep process (18), hetero-VISA and, ultimately, VISA may be selected from just such a population of isolates that display heterogeneously reduced susceptibility to vancomycin but do not meet criteria for hetero-VISA. We studied patients with MRSA bacteremia in our institutions to determine the prevalence, among the infecting strains, of hetero-VISA, and of non-hetero-VISA isolates that nevertheless exhibited varying degrees of susceptibility to vancomycin among subpopulations. Additional objectives were to identify factors predictive of bacteremia with such isolates and to determine whether bacteremia with such isolates affected patient outcomes.

Methods

Microbiologic Methods

Bacterial Isolates

MRSA bloodstream isolates that had been stored nonselectively at Beth Israel Deaconess Medical Center from September 1998 through November 2001 and nosocomial bloodstream isolates from patients in intensive care units at Johns Hopkins Hospital from July 1997 through April 2000 were used. In addition, the following strains were used as controls: ATCC 29213 (Methicillin-susceptible *S. aureus*), ATCC 33591 (vancomycin-susceptible MRSA), ATCC 51299 (vancomycin-resistant *Enterococcus faecium*) and PC3 (VISA strain contributed by A. Tomasz) (19).

Screening for Heterogeneously Reduced Susceptibility to Vancomycin

Suspensions of 0.5 McFarland turbidity standard in brain heart infusion (BHI) broth were prepared from isolates after overnight incubation. Ten microliters of each suspension was injected onto BHI agar plates containing 4 mg/L vancomycin. Because of the reported inducibility of vancomycin heteroresistance in some strains of *S. aureus* by NaCl (13), each isolate was screened on agar with and without 4% NaCl supplementation.

Plates were incubated at 35°C. Results were recorded after 24 h and 48 h of incubation. For any growth in excess of a single pinpoint colony on screening media, either with or without 4% NaCl supplementation, a positive result was recorded.

Susceptibility Testing

Susceptibility to vancomycin was determined by agar dilution, according to NCCLS guidelines (17), with Mueller-Hinton agar. Testing was performed on all MRSA bloodstream isolates (parent isolates), as well as on those colonies that grew on screening media (subclones). MIC testing was performed on colonies taken from the screening agar on which they exhibited optimal growth (i.e., BHI agar containing 4 mg/L vancomycin, with or without 4% NaCl).

Identity Confirmation and Strain Typing

The identity of parent isolates and subclones was confirmed by Gram stain, catalase testing (20), and latex agglutination testing (Staphaurex, Murex Biotech, Ltd., Dartford, UK). Strains were grouped by type and subtype by using pulsed-field gel electrophoresis (PFGE) (21). Plugs were made by using standard techniques. Macrorestriction was performed with *Sma*I (22).

Population Analysis

Suspensions of 3.0 McFarland turbidity standard in BHI broth were made from overnight cultures of selected parent isolates and subclones. Subclones were grown in 4 mg/L vancomycin before suspension in broth. Seven serial 10-fold dilutions of each suspension were prepared. Twenty-five microliters of each suspension and dilution was injected twice onto BHI agar containing 4% NaCl, at vancomycin concentrations of 0 mg/L, 1 mg/L, 2 mg/L, 4 mg/L, and 8 mg/L. NaCl was added due to the enhanced growth we observed on NaCl-containing media during screening. Colonies were counted after 48 h of incubation at 35°C, and sums of each inoculum pair were averaged.

Epidemiologic Methods

Clinical Data Collection and Inclusion Criteria

For the epidemiologic analysis, the unit of observation shifted from the bacterial isolate to the patient with bacteremia. Demographic and clinical data for the patients with saved MRSA bloodstream isolates were collected from electronic medical records and from hospital databas-

es. A patient could be included in the cohort only once, regardless of the number of isolates generated during the study period. Patients with multiple isolates were included at the time MRSA was first isolated from the bloodstream and the isolate grew on screening media. Those patients whose isolates never grew on screening media were included at the time of their first MRSA bloodstream isolation. Inpatients only were considered in the analysis. The study was approved by the ethics review boards of Beth Israel Deaconess Medical Center and of Johns Hopkins Hospital and performed at both institutions.

Study Design

We conducted a two-part retrospective analysis on patients with MRSA bacteremia: a case-control analysis, which considered the clinical features at the time of culture, and a cohort analysis, which evaluated outcomes after culturing.

For the case-control study, patients whose MRSA bloodstream isolates exhibited growth on screening media (case-patients) were compared with patients whose isolates exhibited no growth (controls), in terms of the following features: age, sex, coexisting chronic conditions, hospital events, and antibiotic exposures before culture. Particular attention was given to exposure to vancomycin, examined both as a dichotomous variable and for cumulative days of exposure.

In the cohort study, we compared the above two groups of participants for the following postculture outcomes: deaths, discharge disposition, and duration of hospital stay after culture. Possible confounding variables were evaluated through multivariate modeling.

Statistical Analysis

Statistical analyses were performed by using SAS statistical software (version 8e, SAS Institute, Inc., Cary, NC). Continuous variables were compared by using the Student t test or the Wilcoxon rank sum test, depending on the normality of the distribution. Binary variables were compared by using the Fisher exact test. Nonbinary categorical variables were compared by using the chi-square test. Multivariate logistic regression models were used in the death and disposition analyses to control for confounding. A Cox proportional hazards model was used in the time-to-discharge analysis; observations were censored at patient's death. For all statistical tests, a p value of ≤ 0.05 was considered significant.

Results

Microbiologic Results

We tested 173 MRSA bloodstream isolates from 154 patients. For the following reasons, we excluded 24 isolates from the analysis: 19 represented additional isolates from patients already included in the cohort, 4 came from outpatients, and 1 came from a patient whose hospital stay extended beyond the study period. Thus, we evaluated 149 isolates, each cultured from the blood of a unique inpatient, and will describe them here.

All isolates were susceptible to vancomycin (MIC_{50}, 1 mg/L; MIC_{90}, 1 mg/L; range 0.5–2 mg/L). Isolates from 61 patients (41% of the patient cohort) grew on screening media within 48 h. Subclones of 46 of these isolates (75%) exhibited a two- to fourfold increase in MIC compared with the parent strain. No subclones, however, exceeded the 4 mg/L NCCLS breakpoint for vancomycin susceptibility (the MIC of vancomycin for all but one subclone was $\leq$2 mg/L; for one subclone it was 4 mg/L) (1). We therefore did not characterize any of the isolates as hetero-VISA. We characterized isolates with positive results on our screening assay as having heterogeneously reduced susceptibility to vancomycin.

The isolates comprised 11 different PFGE types. We assigned letters and numbers to PFGE types and subtypes, respectively. One hundred and three (69%) were variants of type A, which had 35 different subtypes. Twenty-two (15%) were variants of type B, which had seven different subtypes. The remaining 24 isolates (16%) consisted of nine different types. Type A was associated with the absence of heterogeneously reduced susceptibility (odds ratio [OR] 0.35; p=0.004), whereas type B was associated with its presence (OR 4.86; p=0.002). The results of strain typing and the relationship between type and screening results are summarized in Table 1. Figure 1 shows the results of PFGE performed on several isolates.

We performed population analysis on eight isolates (four that exhibited growth on screening media and four with no growth) to determine the utility of population analysis in distinguishing between these two types of isolates. The results, represented graphically in Figure 2a, do not enable such a distinction to be made. A shift in the curve is apparent, however, for two isolates with positive screening results from the same patient cultured 14 months apart. For the earlier isolate, the MIC of vancomycin was 0.5 mg/L. For the later isolate, cultured after several interval admissions for MRSA bacteremia in which the patient received vancomycin, the MIC was 1.0 mg/L, and an upwardly shifted population curve (signifying a greater proportion of the population with higher MICs of vancomycin) was observed. A subsequent population analysis, run on selected parent-subclone pairs for isolates that grew on screening media, demonstrated an upward shift in the population curve for the subclone versus the parent when a corresponding increase in MIC existed. A representative population curve is shown in Figure 2b.

Table 1. Strain typing and relationship to reduced vancomycin susceptibility screening results

	No. isolates	Positive results on screening	Odds ratio (95% confidence interval)	p
Type A	103	34 (33%)	0.35 (0.17 to 0.71)	0.004
Type B	22	16 (73%)	4.86 (1.87 to 13.29)	0.002
Other type	24	11 (46%)	-	-

Epidemiologic Results

A comparison of patients with isolates with positive screening results (cases) to those with negative results (controls) demonstrated no significant differences in terms of age, sex distribution, coexisting chronic conditions, recent hospitalization, or hospital events before culture (Table 2). Similarly, no differences were found between the groups with respect to administration of any antibiotic, administration of vancomycin specifically, or number of days of vancomycin exposure before culture (Table 3). In the cohort analysis of the impact of the screening phenotype on patient outcomes, no differences were noted between case-patients and controls in the proportion who died during hospitalization (p=1.00; adjusted for severity of illness, p=0.98) or discharge disposition (Table 4). Similarly, for patients who survived until discharge, the time to discharge did not differ between the two groups (hazard ratio 0.99; 95% confidence interval, 0.67 to 1.47; p=0.97) after controlling for duration of stay prior to culture.

Given the absence of standardized criteria for determining the screening phenotype, we further analyzed the data using a number of more stringent definitions. If we considered growth on screening media within 48 h with an associated increase in MIC among the subclones as the criteria for a positive screening result (n=46), again no significant predictors of the phenotype and no differences in outcomes were found. Twenty patients (13%) had isolates that grew on screening media within 24 h. Using this characteristic as the screening criterion, we found that the only significant predictor of this phenotype was intensive care unit stay before culture (OR 2.95; 95% confidence interval 1.07 to 8.15; p=0.05); no differences in outcomes were found (data not shown).

Finally, since the criteria for isolate collection were not identical in the two study institutions, we performed a subgroup analysis using data from Beth Israel Deaconess Medical Center patients alone (n=120). This analysis did not change the results using the 48-h growth cutoff. Analysis of these data using the 24-h cutoff showed diabetes mellitus to be the only significant predictor of heterogeneously reduced susceptibility to vancomycin (OR 3.52; 95% confidence interval, 1.04 to 11.96; p=0.05), with no differences in outcomes (data not shown).

Discussion

First reported in a clinical specimen from Japan in 1997 (2), VISA strains (MIC 8–16 mg/L) (17) have now been isolated in numerous countries around the world (3,24,25) and have been identified in several patients in the United States (7,19,26). These patients have in common extensive prior exposure to vancomycin, and their clinical courses are notable for a suboptimal response to this agent (1,7). Since VISA isolates are presumed to spread as do vancomycin-susceptible strains of *S. aureus*, their appearance has prompted the issuance of guidelines for their identification and control of transmission (27,28).

Shortly after the report of the first VISA isolate was published, Hiramatsu et al. reported an *S. aureus* isolate exhibiting heteroresistance to vancomycin (6). They found this phenotype of vancomycin heteroresistance to be widespread in Japanese university hospitals. In the ensuing 5 years, reports emerged of heteroresistant *S. aureus* in numerous countries (6–16).

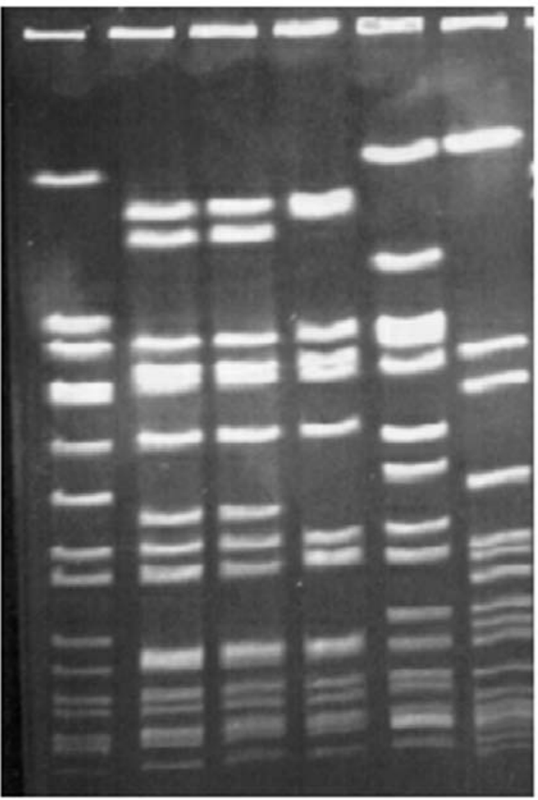

Figure 1. Pulsed-field gel electrophoresis of selected isolates, demonstrating predominant and secondary types. Lane 1, *Staphylococcus aureus* NCTC 8325, used as DNA molecular weight reference marker; lanes 2 and 3, clinical isolates of type A13; lane 4, clinical isolate of type A1; lane 5, clinical isolate of type B2; lane 6, clinical isolate of type H.

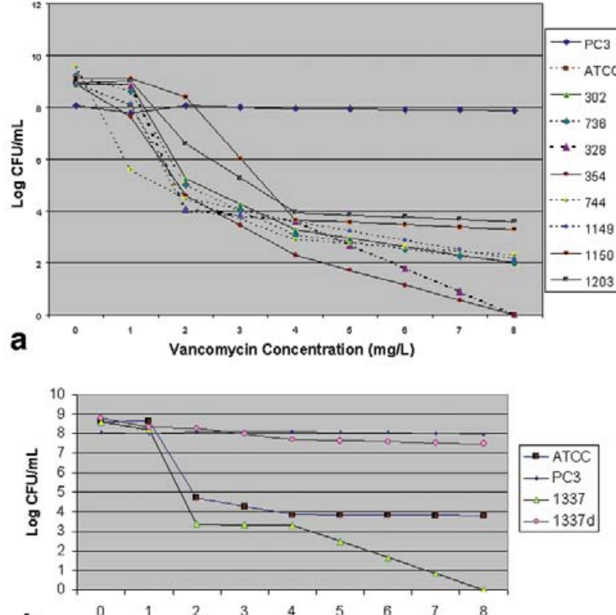

Figure 2. a) Population analysis of parent isolates and reference strains. PC3, vancomycin-intermediate *Staphylococcus aureus* (VISA) reference strain (published vancomycin MIC 8 mg/L (19); MIC 4 mg/L by our assay); ATCC, methicillin-resistant *S. aureus* (MRSA) reference strain ATCC 33591 (MIC 1 mg/L by our assay); clinical isolates exhibiting growth on screening media (MIC in parentheses, in mg/L, followed by MIC of subclone)—302 (1, 4), 354 (0.5, 1), 1150 (1,2), 1203 (1,2); clinical isolates with no growth on screening media (MIC in parentheses, in mg/L)—328 (1), 736 (1), 744 (1), 1149 (1). Isolates with growth on screening media are represented by solid lines; isolates with no growth are represented by broken lines. Isolates 354 and 1150 were cultured from the same patient, 14 months apart. b) Population analysis of an MRSA bloodstream isolate exhibiting heterogeneously reduced susceptibility to vancomycin, and its subclone, compared with reference strains. ATCC, MRSA reference strain ATCC 33591 (MIC 1 mg/L by our assay); PC3, VISA reference strain (published MIC 8 mg/L (19); MIC 4 mg/L by our assay); 1337, clinical isolate (MIC 1 mg/L); 1337d, subclone of 1337 grown on screening media (MIC 2 mg/L). While the population curve representing 1337d closely resembles that of PC3, this subclone did not meet MIC criteria for VISA. CFU, colony-forming units.

Several areas of uncertainty have marked the subject of vancomycin heteroresistance since it was first described. First, no standardized definition exists, and investigators have defined it by using a variety of criteria (6,8,11,16,29,30). Second, the clinical significance of heteroresistance remains unclear. Although existing evidence supports the hypothesis that heteroresistant isolates have a greater likelihood of developing homogeneous intermediate resistance than do susceptible strains (6), and other data suggest an association between isolation of VISA and an adverse outcome (7), few studies have examined whether specific risk factors exist for infection with hetero-VISA, and whether such infection is associated with adverse outcomes. Those studies that have examined risk factors have had small sample sizes (10), inadequate generalizability (8), or a method of control selection that did not allow for direct comparison between patients with hetero-VISA and those infected with *S. aureus* isolates that were homogeneously susceptible to vancomycin (13). No study before ours has explored the clinical implications of varying degrees of vancomycin susceptibility in MRSA isolates that do not qualify as hetero-VISA.

This study addresses the prevalence of vancomycin heteroresistance among bloodstream MRSA isolates at two large, urban teaching hospitals and explores clinical correlates of bacteremia with isolates exhibiting heterogeneously reduced vancomycin susceptibility, as defined by growth on vancomycin screening agar (vancomycin 4 mg/L). We chose a screening method to encompass as broad an array of potentially heteroresistant isolates as possible. By subjecting subclones to agar dilution, however, we were deliberately conservative, in order to determine whether our isolates fulfilled Hiramatsu's criteria for vancomycin heteroresistance in as unambiguous a manner as possible (6). Although none of our isolates were characterized as hetero-VISA under these strict criteria, over 40% had subpopulations capable of growth on our vancomycin-containing screening media. Such findings are plausible because *S. aureus* strains are known to differ in their propensity to develop reduced susceptibility to vancomycin (31). It is possible that some or all of the isolates from our cases are potential precursors of truly heteroresistant isolates (hetero-VISA), which may in turn be forerunners of VISA (7,18,32).

Howe et al. have criticized the method of screening for vancomycin heteroresistance in the presence of the antibiotic, followed by MIC testing of subclones, as being poorly reproducible and potentially selecting for, rather than simply detecting, resistant mutants (29). Regarding reproducibility, we repeated screening for 12 clinical isolates (6 with initially positive results, and 6 with negative results) at different times, using freshly made media each time. Ten (83%) of these had concordant results for the two tests (growth within 48 h on screening media with or without NaCl was the criterion for a positive result). Regarding the possibility of selection, even if this method does select for subclones that grow on screening media, its ability to do so may represent detection of a strain-specific phenomenon that could be of clinical importance if it occurs in vivo during vancomycin therapy.

Having identified the positive screening phenotype among our isolates, we sought to uncover clinical predictors of bacteremia with isolates exhibiting this phenotype, as well as to determine whether such bacteremia was associated with adverse outcomes. Our results were negative on both counts. The results were rendered more robust by

Table 2. Descriptive characteristics of cohort

Characteristic	Cases, n=61 (%)	Controls, n=88 (%)	p
Mean age (y)	61±17	64±14	0.21
Male	37 (61)	53 (60)	1.00
Diabetes mellitus	30 (49)	34 (39)	0.24
Renal disease	20 (33)	29 (33)	1.00
Hemodialysis	10 (16)	12 (14)	0.65
Cardiovascular disease	41 (67)	56 (64)	0.73
Pulmonary disease	19 (31)	35 (40)	0.30
Hepatic disease	8 (13)	7 (8)	0.41
HIV	3 (5)	3 (3)	0.69
Prosthetic joint or valve, or permanent pacemaker	13 (21)	11 (13)	0.18
Hospitalization at same institution ≤30 days preceding culture	51 (84)	66 (75)	0.23
Surgery during admission before culture	17 (28)	20 (23)	0.56
Intensive care unit stay during admission before culture	33 (54)	38 (43)	0.24
Severity of illness score[a]			0.62

[a]Severity of illness is based on modified McCabe criteria (23): 1, severe underlying coexisting chronic condition, at imminent risk of death; 2, significant underlying coexisting chronic condition, not at imminent risk of death; 3, no significant underlying coexisting chronic condition.

remaining essentially unchanged even as we varied the definition of a positive screening result and performed an institution-based subgroup analysis. More stringent definitions of positivity other than those we explored (for example, a requirement of growth on non–salt-containing media), may have led us to undiscovered clinical differences between case-patients and controls.

These negative results can be interpreted in a number of ways. First, our study may have lacked statistical power to detect small differences between the groups in predictors and outcomes. Given the degree to which most of the p values deviate from statistical significance, however, analysis of a substantially larger cohort would be required to disprove such a claim. Moreover, our isolate cohort was highly clonal, as a single type accounted for 69% of isolates and the two most prominent types accounted for 84%. This degree of clonality among pathogenic isolates of MRSA may bias any attempted comparison of clinical features among the bacteremic patients (33).

Another possible explanation for our failure to detect outcome differences between case-patients and controls is the small degree of vancomycin exposure among all patients in the cohort before blood was drawn for culture. Only 17% (10 case-patients and 14 controls) of patients in the case and control groups received vancomycin before their blood was cultured, and the average amount of time that vancomycin was used by case-patients and controls before culture was approximately 1 day. Clinically, this finding is understandable: Most patients in the cohort had no reason to receive vancomycin before the growth of MRSA. Still, as pharmacologic data were not uniformly available before the patient's hospitalization, we were able to focus on receipt of vancomycin during the index admission only, thereby perhaps limiting our ability to distinguish between study groups based on vancomycin exposure.

No difference may actually exist between bacteremia with VSSA strains that exhibit heterogeneously reduced susceptibility to vancomycin, as defined by growth on vancomycin 4 mg/L screening agar, and homogeneously susceptible isolates. Such a conclusion would not rule out a clinical difference associated with hetero-VISA bacteremia; because our cohort contained no such cases, we cannot draw any conclusions regarding this question. Also, we may have not detected a difference in outcomes because we did not focus on the relevant ones. If, for example, heterogeneously reduced vancomycin susceptibility comes at the expense of a certain degree of virulence, as suggested by Burnie et al. on the basis of a mouse model (34), then we would not necessarily expect our case-patients to have more hospital deaths or even a greater length of stay in the hospital. We might, however, expect to find several years from now that case-patients will have a greater number of recurrent MRSA infections than will controls, or longer durations of bacteremia during such infections, due to failure of vancomycin to eradicate the organism effectively.

Our results show that despite a subtle phenotypic difference in the MRSA isolates of a large minority of the patients in our cohort, these patients were no different than the reference group with respect to clinical characteristics, antimicrobial use and other hospital exposures, and clinical outcomes. These results add weight to assertions that

Table 3. Antibiotic exposures during hospitalization before culture

Antibiotic exposure	Cases n=58 (%)	Controls n=84 (%)	p
Administration of any antibiotic	27 (47)	45 (54)	0.50
Administration of vancomycin	10 (17)	14 (17)	1.00
Number of days of receiving vancomycin (mean)	1.2±4.8	1.0±3.5	0.88

Table 4. Discharge disposition

Disposition	Case-patients, n=61 (%)	Controls, n=87 (%)	p (unadjusted)	p (adjusted for severity of illness)
Discharged to home	13 (21)	22 (25)		
Discharged to an institution	32 (52)	42 (48)	0.83	0.67
Died during hospitalization	16 (26)	23 (26)		

clinical microbiology laboratories need not routinely screen for vancomycin heteroresistance in *S. aureus* isolates with vancomycin MICs in the susceptible range (2,8). Additional studies with larger cohorts and a longer period of follow-up are needed to validate these findings, determine whether they apply to infection with true hetero-VISA, and evaluate outcomes suggestive of noneradicated, indolent infection.

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Serogroup W-135 Meningococcal Disease during the Hajj, 2000

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An outbreak of serogroup W-135 meningococcal disease occurred during the 2000 Hajj in Saudi Arabia. Disease was reported worldwide in Hajj pilgrims and their close contacts; however, most cases were identified in Saudi Arabia. Trends in Saudi meningococcal disease were evaluated and the epidemiology of Saudi cases from this outbreak described. Saudi national meningococcal disease incidence data for 1990 to 2000, were reviewed; cases from January 24 to June 5, 2000 were retrospectively reviewed. The 2000 Hajj outbreak consisted of distinct serogroup A and serogroup W-135 outbreaks. Of 253 identified cases in Saudi Arabia, 161 (64%) had serogroup identification; serogroups W-135 and A caused 93 (37%) and 60 (24%) cases with attack rates of 9 and 6 cases per 100,000 population, respectively. The 2000 Hajj outbreak was the first large serogroup W-135 meningococcal disease outbreak identified worldwide. Enhanced surveillance for serogroup W-135, especially in Africa, is essential to control this emerging epidemic disease.

Meningococcal disease, caused by the bacterium *Neisseria meningitidis*, results in meningitis and sepsis in persons of all ages. The disease has a case-fatality rate of at least 10%, and chronic sequelae occur in 12% to 15% of survivors (1). Among the 13 meningococcal serogroups defined by serologic reactivity of the meningococcal capsular polysaccharide, serogroups A, B, and C are most commonly associated with disease worldwide, while other serogroups (e.g., X and Y) are of increasing importance (1,2). Although most meningococcal disease is sporadic, outbreaks also occur, historically caused by serogroups A, B, and C (1). Outbreaks can vary in magnitude, depending on many factors, including the serogroup and molecular characteristics of the epidemic strain. Serogroup A outbreaks in the "meningitis belt" of sub-Saharan Africa reach incidence rates of hundreds of cases per 100,000 population during a single dry season (3–6); serogroup B outbreaks are characterized by increased rates of disease occurring over years (7–9); and serogroup C outbreaks usually involve smaller numbers of cases occurring over weeks to months (10,11). Molecular characterization of *N. meningitidis* isolates has identified major electrophoretic-type (ET) complexes, such as ET-5 (7–9), and the ET-37 complex (12), as primarily associated with outbreaks of serogroups B and C disease, respectively.

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In 1987, a meningococcal outbreak was associated with the Hajj pilgrimage (13). This pilgrimage, one of the central religious duties of Islam, draws 1–2 million Muslims from around the world to Saudi Arabia. The 1987 outbreak was caused by a strain of *N. meningitidis* serogroup A termed subgroup III. This subgroup was first associated with an outbreak in Nepal in 1983 and 1984 and after the 1987 Hajj outbreak caused massive meningococcal outbreaks in sub-Saharan Africa (4,14) with incidence rates from 250 to 1,000 cases/100,000 persons and at-risk populations numbering in the millions (5,6).

In response to the 1987 outbreak, Saudi Arabia required proof of meningococcal vaccination to issue Hajj pilgrimage visas (15). Bivalent (serogroup A+C) polysaccharide meningococcal vaccine is commonly used to meet this requirement (16); however, a quadrivalent serogroup A/C/Y/W-135 vaccine is used in some countries, particularly the United States (17). Vaccination campaigns with bivalent meningococcal vaccine have also been the principal strategy for controlling serogroup A outbreaks in Africa (18,19).

During the 2000 Hajj, an outbreak of meningococcal disease principally involved serogroup W-135 (17,20,21), an uncommon cause of disease that accounts for <2% of cases worldwide (2). Cases were reported in returning pilgrims in countries throughout the world including Europe, the United States, Asia, Africa, and the Middle East (17,21,22); however, most cases were identified in Saudi Arabia. The carriage and transmission characteristics of serogroup W-135 are not well understood. Furthermore, bivalent meningococcal vaccine cannot protect against serogroup W-135 disease. Therefore, if serogroup W-135 causes large outbreaks in Africa, as subgroup III did during the last decade, the practical implications will be serious. We evaluated recent trends of meningococcal disease

in Saudi Arabia and examined demographic and clinical characteristics of the 2000 Hajj meningococcal cases in Saudi Arabia to assess the global impact of serogroup W-135 outbreaks.

Materials and Methods

Surveillance

Since 1990, the Saudi Arabian Ministry of Health has conducted national surveillance for meningococcal disease. From 1995 through 2000, the Ministry of Health collected data for the number of cases by serogroup and month; for 1990 through 1995, only national serogroup-specific totals by year were available.

During the 5-day Hajj pilgrimage, pilgrims complete a 24-mile round-trip journey from Mecca through the Plain of Arafat; many pilgrims subsequently perform additional ritual activities in Medina. During the 2 weeks before and 1 week after Hajj, Ministry of Health officials move meningococcal surveillance operations to Mecca and coordinate with regional teams conducting active surveillance for meningitis through daily contact with local hospitals, laboratories, and public health personnel in Mecca, Medina, and Jeddah. Surveillance data include demographic information, results of blood and cerebrospinal fluid (CSF) culture, serogroup identification of meningococcal isolates, and latex agglutination of CSF. Since most cases of meningococcal disease in the 2000 outbreak were reported in Mecca and Medina and since most Hajj pilgrims fly into the country through Jeddah, we focused our outbreak investigation on these three cities. The timing of the pilgrimage is based on the Islamic lunar calendar; the 2000 Hajj pilgrimage was March 14–19. We reviewed records for suspected meningococcal disease cases (see definition below) identified in Mecca, Medina, and Jeddah from January 21 through June 5, 2000. Demographic, microbiologic, and clinical data were collected from records compiled by the Ministry of Health and regional health directorates and through review of clinical laboratory records and inpatient charts from all hospitals serving the three regions.

Eighty-nine cases of serogroup W-135 disease were also reported to the World Health Organization from countries outside Saudi Arabia in association with pilgrims returning from the 2000 Hajj (22,23); additional information on these cases was not collected.

Definitions

A case of meningitis was suspected for patients with fever (rectal temperature ≥38.5°C or axillary temperature >38.0°C) and stiff neck (or bulging fontanel for patients ≤1 year of age). Confirmed meningococcal disease was defined as culture of *N. meningitidis* from blood or CSF, or detection of *N. meningitidis* antigen by latex agglutination of CSF from a person with suspected meningococcal disease in Mecca, Medina, or Jeddah from January 21 to June 5, 2000. Meningococcal meningitis was defined as a case of confirmed meningococcal disease with evidence for *N. meningitidis* in CSF through culture or latex agglutination; CSF specimens were evaluated for all cases reported here.

A patient was identified as a Saudi Arabian resident (Saudi or non-Saudi citizenship) if medical or surveillance records indicated that he or she resided in Saudi Arabia. Patients were defined as Hajj travelers if Ministry of Health or medical records indicated they had officially entered the country to perform Hajj or Umrah (a pilgrimage to Mecca that occurs year-round but is frequently performed by Hajj pilgrims just before Hajj); visitors without clear documentation were categorized as nonofficial. We defined Hajj pilgrims to include all persons who attended Hajj, based on Ministry of Health statistics. Severe illness was defined as admission to intensive care or death; chronic illness denoted a history of diabetes, hypertension, or heart disease. Meningococcal vaccination during the previous 3 years was recorded on Saudi Ministry of Health case report forms, which were typically obtained from the patient; vaccination status was not confirmed.

Laboratory Analysis

N. meningitidis was identified on the basis of World Health Organization recommendations (24). Meningococcal serogroup identification was performed in the hospital clinical laboratory by latex agglutination for specific polysaccharide capsular types (A, B, C, Y, and W-135) on all available isolates with Wellcogen bacterial antigen kits (Murex Diagnostics Ltd., Dartford, U.K.); isolates were not retained in long-term storage. Cases without serogroup identification were categorized as serogroup unknown; this classification most commonly arose from lack of a viable isolate or unavailability of serogroup analysis kits. When the serogroup identified in case records conflicted with laboratory records, the serogroup identified in the hospital clinical laboratory log was used. Two serogroup W-135 and two serogroup A isolates collected from patients in Saudi Arabia were molecularly characterized by multilocus enzyme electrophoresis (MLEE) (25), serotyping/serosubtyping (26), pulsed-field gel electrophoresis (PFGE) (27), and multilocus sequence typing (28). Complete molecular subtyping of all serogroup W-135 isolates collected during the 2000 Hajj outbreak from patients identified in Saudi Arabia and from returning pilgrims whose illness was diagnosed in their country of origin are reported elsewhere (29). No serogroup W-135 isolates from Saudi cases identified before the 2000 Hajj were available for molecular characterization.

Attack Rate Calculations

We calculated serogroup A and serogroup W-135 attack rates by using the official Saudi Ministry of Health estimate of 1.7 million Hajj attendees (30) as the denominator; the number of Hajj attendees by nationality was not available. For the serogroup W-135 attack rate, we combined the number of laboratory-confirmed serogroup W-135 cases with the estimated number of meningococcal disease cases of unknown serogroup attributed to serogroup W-135. The latter figure was estimated by starting with the total number of cases in which serogroup was identified, calculating the proportion of these attributed to serogroup W-135, and multiplying this proportion by the total number of cases in which serogroup was not identified. A similar procedure was used to calculate the serogroup A attack rate. Calculations, which were based on the serogroup distribution by region or for the outbreak as a whole, yielded similar results. We could not assess Hajj attendance of Saudi residents, and some patients may have been residents who did not attend Hajj; therefore, these calculations may overestimate the attack rate. The estimated 90 patients with serogroup W-135 disease identified in other countries associated with the 2000 Hajj (22,23) were not included in this analysis because of the lack of information identifying which cases arose through secondary transmission in the pilgrim's country of origin after the Hajj.

Statistical Analysis

Univariate analysis of the retrospective surveillance data was performed in Epi6 and EpiInfo 2000 (CDC, Atlanta, GA) and SAS 8.1 for Windows (SAS, Inc., Cary, NC) with the Fisher exact test with Mantel-Haenszel odds ratios (OR) to assess the association of individual variables with W-135 disease or severe illness. Demographic characteristics of cases with known versus unknown serogroup differed only in the high proportion of unknown serogroup cases identified in Jeddah. The category of unknown serogroup was treated as a missing value for all statistical analysis involving serogroup-specific data except attack rate calculations. Given the small number of cases in Jeddah and the similar serogroup-specific case distribution in Mecca, the variable identifying the metropolitan region where the case was diagnosed was divided into Medina versus Jeddah and Mecca.

All variables with $p \leq 0.1$ on univariate analysis, as well as potential confounders, were entered into logistic regression. Stepwise, multivariate logistic regression was performed to assess risk factors associated with developing serogroup W-135 compared to either serogroup A disease or to all cases with known serogroup. Separate multivariable models were used to evaluate risk factors for severe illness and fatal disease.

Results

From 1995 through 1999, the median annual number of meningococcal disease cases in Saudi Arabia (population 20 million) was 42 (range 20–107) with a resulting national annual meningococcal disease incidence of 0.2 cases/100,000 population (Figure 1). From 1995 to 1998, a median of 10 cases (range 4–20) occurred within 1 month of the Hajj. Most cases were serogroup A or W-135 disease; however, in 1998 a cluster of 20 cases of serogroup B meningococcal disease was identified. In 1997, a total of 72 cases of meningococcal disease, predominantly serogroup A, were reported in association with Umrah before the Hajj.

Serogroup W-135 has been present to a notable degree in Saudi Arabia at least since 1990. During 1995 through 1999, serogroup W-135 disease accounted for 13% of all meningococcal disease in Saudi Arabia (Figure 1). Saudi serogroup-specific surveillance data for 1990 through 1994 are available only by Islamic calendar year and not by month; during this time, serogroup W-135 accounted for 1.5% to 54% of annual meningococcal disease with the peak (24 cases) occurring in 1990–1991.

In the year 2000, retrospective review identified 264 suspected cases of meningococcal disease in Mecca, Medina, or Jeddah. Of these, 253 (96%) were laboratory confirmed with 179 (71%) positive by CSF or blood culture and 74 (29%) negative by culture but positive by CSF latex agglutination. Seventy patients died, for a case-fatality rate of 28%. Cases increased during the month preceding the Hajj (Figure 2), peaking just after the Hajj ended; the last case was identified on June 5. The median age of patients with confirmed meningococcal disease was 40 years; most were male (Table 1). Overall, 56% of patients were Hajj travelers. Persons identified as Hajj travelers were older (median age 50 years vs. 5 years, $p<0.001$), more likely to be male (OR=1.9, p=0.02), and more likely to have a chronic illness (OR=7.8, $p<0.001$) than residents.

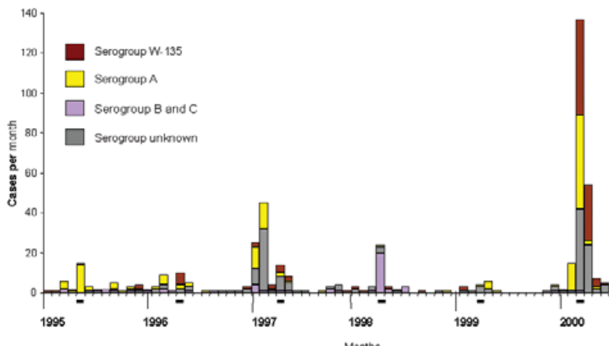

Figure 1. Meningococcal disease in Kingdom of Saudi Arabia, by month, 1995–2000. Cases of meningococcal disease with dates converted from Islamic calendar months. The period of the Hajj pilgrimage for each year is underscored.

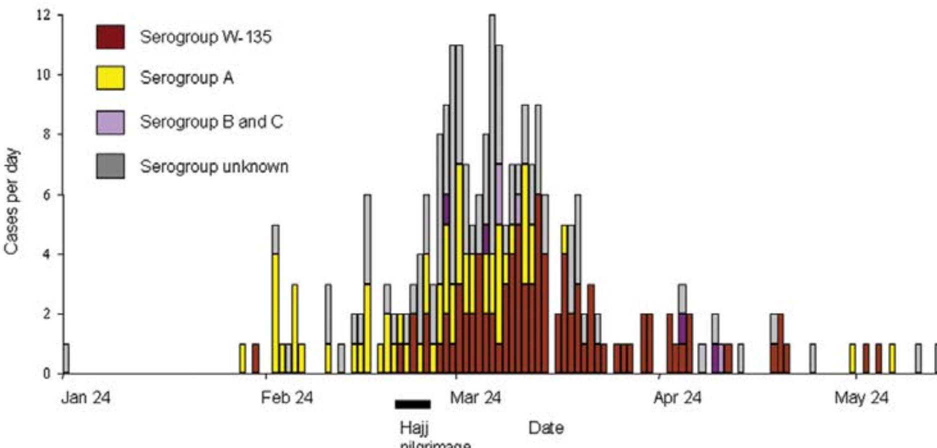

Figure 2. Meningococcal disease during the 2000 Hajj: Jeddah, Mecca, and Medina, January 24–June 5, 2000. The number of cases of serogroup-specific meningococcal disease is shown by date. The duration of the 2000 Hajj is indicated.

A serogroup was identified for 161 (64%) of the 253 confirmed cases. Ninety-three cases (37% of all confirmed cases) were attributed to serogroup W-135 and 60 (24%) to serogroup A. Both serogroup A isolates from the 2000 outbreak were confirmed as subgroup III (ET-734, ST-5 with PFGE patterns characteristic for the "first pandemic wave" (14); serogroup W-135 isolates from the 2000 outbreak belonged to the ET-37 complex, as defined by MLEE, and were designated as the (W)ET-37 clone (29). Serogroup W-135 case-patients had a median date of admission 2 weeks later than for serogroup A patients (p<0.001) (Table 1). The diagnosis for most serogroup A patients was made in Mecca; serogroup W-135 cases were diagnosed in patients in both Mecca and Medina. Rates of meningococcal vaccination did not differ between serogroup W-135 and serogroup A case-patients (67% vs. 59%, respectively; p=0.35); however, among Hajj travelers, patients with serogroup W-135 were more likely to have received meningococcal vaccine than were those with serogroup A disease (93% vs. 77%, p=0.05). The calculated attack rates of serogroup W-135 and serogroup A disease were 8.9 and 5.8 cases/100,000, respectively. During the month when the outbreak peaked, weekly serogroup W-135 attack rates remained ≤1 case/100,000 persons. Multivariable modeling for risk factors for serogroup W-135 disease showed that, after age was controlled for, patients with cases diagnosed in Medina were at increased risk for serogroup W-135 compared with all other cases with known serogroups

Table 1. Demographic characteristics of patients with confirmed meningococcal disease, Saudi Arabia[a]

Feature	All cases, n=253 (%)	Serogroup-specific cases, n (%) W-135, n=93	A, n=60
Median date of hospital admission (range)	Mar 29 (Jan 24–June 2)	Apr 5 (Feb 22–May 24)	Mar 22(Feb 20–May 26)
Female[b]	118 (47)	48 (52)	20 (33)
Median age, y (range)	40 (0.2 to 80)	35 (0.2 to 80)	42 (2 to 75)
No. of cases identified in Jeddah	41 (16)	3 (3)	3 (5)
No. of cases identified in Mecca[b]	158 (62)	53 (57)	50 (83)
No. of cases identified in Medina[b]	54 (21)	37 (40)	7 (12)
National status[b]			
Resident (Saudi/non-Saudi background)	98 (39)	43 (46)	17 (29)
Hajj or Umrah traveler	143 (57)	48 (52)	42 (71)
Nonofficial	11 (4)	2 (2)	0 (0)
Chronic illness	44 (17)	12 (13)	10 (17)
History of any meningococcal vaccination	130/222 (59)	55 (67)	30 (59)
National origin			
Africa	89 (35)	36 (39)	19 (32)
Asia	106 (42)	31 (33)	29 (48)
Southwest Asia[c]	65(26)	17(18)	23(38)
Europe	9 (4)	5 (5)	2 (3)
Middle East	48 (19)	21 (23)	10 (17)

[a]Identified by retrospective surveillance (January 24–June 5, 2000) in Jeddah, Mecca, and Medina. Cases of serogroup B and C disease (four cases each), as well as cases of unknown serogroup, are not shown. Denominator for all confirmed cases is 253 unless stated otherwise (changes in denominators are due to missing values); denominator for serogroup-specific percentages is the total serogroup-specific disease.
[b] Comparison of characteristics for serogroup W-135 versus serogroup A with Fisher exact p value ≤0.05.
[c]Countries included in category of Southwest Asia: Afghanistan, Pakistan, India, Bangaladesh, Myanmar, and Sri Lanka.

Table 2. Clinical characteristics of patients with confirmed meningococcal disease, Saudi Arabia

Feature	All cases, n=253 (%)	Serogroup-specific cases, n (%)	
		W-135, n=93	A, n=60
Seen by clinician ≤1 day after symptom onset	125 (49)	45 (48)	31 (52)
Semiconscious, comatose, or dead[b]	146 (62)	57 (63)	33 (58)
Positive blood culture[b]	72/140	55 (79)	15 (29)
Sepsis without meningitis	23 (9)	19 (20)	4 (7)
Admitted to intensive care[b]	51/212 (24)	27 (35)	7 (14)
>1-day intensive care required[b]	33/202 (16)	19 (26)	3 (6)
Death (case-fatality rate)	70 (28)	27 (29)	16 (27)

[a]Identified by retrospective surveillance (January 24–June 5, 2000, in Jeddah, Mecca, Medina, Saudi Arabia. Cases of serogroup B and C disease (four cases each) as well as cases of unknown serogroup are not shown. Denominator for all confirmed cases is 253 unless stated otherwise (changes in denominators are due to missing values); denominator for serogroup-specific percentages is the total serogroup-specific disease.
[b]Comparison of characteristics for serogroup W-135 versus serogroup A with Fisher exact p value ≤0.05.

(OR=6, 95% confidence intervals [CI] 2.2 to 15.5, p<0.01). While no regional group was at increased risk for serogroup W-135 compared with serogroup A disease, serogroup A disease was more likely to occur in travelers arriving from Southwest Asia than serogroup W-135 disease (OR=2.6, 95% CI 1.1 to 5.6, p=0.02);

We also evaluated clinical characteristics of identified case-patients and found that approximately half were first seen by a clinician ≤1 day after symptom onset; at that time, 62% of case-patients were semicomatose, comatose, or dead. Only 9% of case-patients had sepsis without evidence of meningitis. Patients with serogroup W-135 compared with those with serogroup A were more likely not to have meningitis (OR 3.6, p=0.02) and to be admitted to intensive care (Table 2). Case-fatality rates did not differ by serogroup; patients with chronic illness had a higher case-fatality rate (50% vs. 19%, p=0.0003). For residents and Hajj travelers with serogroup W-135 disease, case-fatality rates were 12% and 46%, respectively (p<0.001). In a multivariate model controlling for age, factors associated with increased risk for fatal disease were the following: admission to intensive care (OR 15, 95% CI 5 to 45), chronic illness (OR 3, 95% CI 1 to 8), and being seen by a clinician ≤1 day of symptoms (OR 2.5, 95% CI 1 to 6); cases diagnosed in Medina were less likely to be fatal (OR=0.2, 95% CI 0.05 to 0.5). In this multivariate model, serogroup was not found to be a significant risk factor for fatal disease. A separate multivariate model for risk factors for severe illness, after age was controlled for, identified serogroup W-135 disease compared with all other cases of known serogroup (OR=2.0, 95% CI 1.2 to 5.2) and being seen by a clinician after ≤1 day of symptoms (OR=2.4, 95% CI 1.2 to 5.1) as associated with increased risk. In this model, persons with a history of chronic illness were at increased risk for severe illness, although this association did not reach statistical significance (OR 2.4, p=0.09).

Discussion

The meningococcal outbreak during the 2000 Hajj was unusual because it consisted of two concurrent outbreaks, one caused by serogroup A and one caused by serogroup W-135. Both outbreaks were detected because the surveillance for meningococcal disease in Saudi Arabia, particularly during the Hajj, is extensive. In the African meningitis belt, serogroups are commonly identified on the basis of analysis of isolates early in the outbreak with little subsequent serogroup evaluation. If this practice had occurred during the 2000 Hajj outbreak, it would likely have been misidentified as an outbreak of solely serogroup A disease; the serogroup W-135 component would have been missed. Longitudinal serogroup-specific surveillance is especially essential for outbreak control with serogroup-specific vaccines.

Although isolates from Saudi Arabia before the 2000 outbreak were not available for analysis, other studies have found that the (W)ET-37 clone caused disease decades before the 2000 outbreak (29). Since serogroup W-135 disease appears to have been endemic in Saudi Arabia for at least the last decade, the (W)ET-37 outbreak strain was likely present in Saudi Arabia before the 2000 Hajj, and this outbreak was probably not due to introduction of a new clone. The (W)ET-37 clone probably not only caused invasive disease but also circulated in the population through nasopharyngeal carriage. In 2001, one study found rare serogroup W-135 carriage in returning pilgrims (31); another study found higher rates of carriage (32). However, the absence of carriage data from Saudi Arabia from 2000 or preceding years makes drawing conclusions about the complicated relationship between carriage and epidemic meningococcal disease in this setting difficult.

Despite previous circulation of the (W)ET-37 outbreak strain, the outbreak described here was the first large W-135 outbreak reported worldwide (33); the attack rate was 9 cases/100,000 population. Although the 2000 serogroup W-135 outbreak was larger than the 2000 serogroup A outbreak, this comparison must be put into the context of other outbreaks. During the 1987 Hajj, 1,841 laboratory-confirmed cases of serogroup A disease (16) were identified in Saudi Arabia among approximately 1.5 million Hajj attendees (34), for an incidence of 120/100,000. The *N.*

meningitidis serogroup A isolates from the 2000 and 1987 Hajj outbreaks are members of the same serogroup A subgroup III, and yet the magnitude of the 2000 outbreak was less than one tenth that of the 1987 outbreak. The requirement for meningococcal vaccination was enacted in Saudi Arabia only after the 1987 Hajj. We found vaccination coverage in the 2000 outbreak case-patients in Saudi Arabia (59% to 67%) similar to coverage reported previously in Mecca residents (16); substantial blunting of serogroup A outbreaks elsewhere has been associated with similar levels of vaccination coverage (5,18). This finding suggests that the potential impact of the serogroup A outbreak during the 2000 Hajj may have also been markedly blunted by vaccination. However, since most vaccination for the Hajj is performed with bivalent, not quadrivalent, vaccine, vaccination coverage for serogroup W-135 during the 2000 Hajj was probably low (35). Therefore, the 2000 outbreak could be interpreted as representing the full outbreak potential of the (W)ET-37 clone within the context of the Hajj—much as the 1987 outbreak represented the unblunted impact of serogroup A subgroup III. Given that the 2000 serogroup W-135 outbreak was very small in comparison to the 1987 serogroup A outbreak, and weekly attack rates associated with the 2000 Hajj outbreak were substantially below the thresholds used to identify meningococcal outbreaks in Africa (19), within the context of the Hajj, the (W)ET-37 clone would appear to be less likely to cause outbreaks with incidence as high as those associated with serogroup A subgroup III. Clearly, this hypothesis is difficult to prove, since a constellation of host, environmental, and pathogen-specific factors in Mecca during the 2000 Hajj likely contributed to propagation of the outbreak strain; we were unable to identify the specific causative factors for this outbreak. A more recent (W)ET-37 outbreak in Burkina Faso, which had epidemiologic features typical of a serogroup A epidemic, may also have been caused by one or more of these factors (36).

Since 1987, a core component for preventing meningococcal disease during the Hajj has been mandatory vaccination of pilgrims and Saudi residents. In 2000, most vaccinated pilgrims received the bivalent vaccine. Given the large number of 2000 Hajj pilgrims and the estimated 85% to 95% vaccine efficacy against serogroup A disease (37,38) (and based on the assumption that reported Hajj pilgrim vaccinations are accurate), the 35 persons who reported having serogroup A disease despite having recently received meningococcal vaccine would be expected vaccine failures. Vaccine failure may have also led to the single primary case of serogroup W-135 disease (39) that occurred among the 11,000 Hajj pilgrims returning to the United States, where quadrivalent vaccine is used. In contrast, 10 primary cases of serogroup W-135 disease were reported among the 10,300 pilgrims returning to the United Kingdom, where bivalent vaccine is used (p=0.01) (20). Since no modifiable risk factors for disease have been identified for the 2000 serogroup W-135 outbreak, quadrivalent vaccine should be used to reduce the risk for serogroup W-135 disease. Given the high overall case-fatality rate of 28% for the 2000 Hajj outbreaks and our finding that chronic illness was a risk factor for severe illness and death, use of quadrivalent vaccine for older and chronically ill pilgrims is a priority. Strategies should also target this population for frequent evaluations for symptoms of disease.

Since 2002, Saudi Arabia has required quadrivalent vaccine for all pilgrims participating in the Hajj (40). However, the higher cost of this vaccine, as well as inadequate global vaccine supply, makes implementing this strategy difficult. If the (W)ET-37 clone continues to cause outbreaks in sub-Saharan Africa, as it did in Burkina Faso in 2002 (36), quadrivalent vaccine would be urgently needed for outbreak management. While empiric use of quadrivalent vaccine for outbreak response in Africa is not warranted at this time, heightened surveillance for serogroup W-135 disease, particularly in the Eastern Mediterranean region and the African meningitis belt, will be essential in designing and assessing future control and prevention strategies for this emergent epidemic disease.

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Molecular Subtyping To Detect Human Listeriosis Clusters

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We analyzed the diversity (Simpson's Index, D) and distribution of *Listeria monocytogenes* in human listeriosis cases in New York State (excluding New York City) from November 1996 to June 2000 by using automated ribotyping and pulsed-field gel electrophoresis (PFGE). We applied a scan statistic ($p \leq 0.05$) to detect listeriosis clusters caused by a specific *Listeria monocytogenes* subtype. Among 131 human isolates, 34 (D=0.923) ribotypes and 74 (D=0.975) PFGE types were found. Nine (31% of cases) clusters were identified by ribotype or PFGE; five (18% of cases) clusters were identified by using both methods. Two of the nine clusters (13% of cases) corresponded with investigated multistate listeriosis outbreaks. While most human listeriosis cases are considered sporadic, highly discriminatory molecular subtyping approaches thus indicated that 13% to 31% of cases reported in New York State may represent single-source clusters. Listeriosis control and reduction efforts should include broad-based subtyping of human isolates and consider that a large number of cases may represent outbreaks.

Listeria monocytogenes is a bacterial foodborne pathogen that can cause severe invasive disease manifestations, including abortion, septicemia, and meningitis. While multiple large outbreaks have been recognized, most cases are thought to be sporadic (1). Human listeriosis is relatively rare, typically includes long incubation periods (7–60 days), usually results in hospitalization (85% to 90%), and often results in death (≤30%) (2). Persons with specific immunocompromising conditions, pregnant women, and newborns appear to be particularly susceptible to invasive listeriosis, and most reported cases occur in these specific risk groups (3,4). Various studies indicate that from 1% to 5% of common ready-to-eat foods may contain *L. monocytogenes* (5–7), and these foods may be widely distributed as a result of current marketing and distribution practices. Traditional epidemiologic surveillance alone may not detect many common source outbreaks, particularly if a limited number of cases occur over a wide geographic area (8,9) because of the unique characteristics of human foodborne listeriosis.

Subtyping methods for *L. monocytogenes* include phenotypic (e.g., serotyping and phage-typing) as well as different DNA-based subtyping methods. Phenotypic methods often yield a low power of discrimination in strains (e.g., >90% of all human isolates represent 3 of the 13 known serotypes), suffer from biologic variability (e.g., phage typing), and may not be applicable to all strains (10). Molecular subtyping methods include multilocus enzyme electrophoresis, ribotyping, pulsed-field gel electrophoresis (PFGE), polymerase chain reaction (PCR), and restriction-fragment length polymorphism (RFLP) analysis. Automated ribotyping was previously used for rapid subtyping *L. monocytogenes* for source tracking, population genetics–based studies, and epidemiologic investigations (11–13); however, it is expensive and not as discriminatory as PFGE (14). PFGE provides sensitive subtype discrimination and is often considered the standard subtyping method for *L. monocytogenes* (15). However, this method is not automated and is labor intensive. Even recently developed rapid protocols take approximately 30 hours to perform (10,15).

We used two molecular subtyping methods (automated *Eco*RI ribotyping and *Asc*I PFGE) to evaluate and compare their discriminatory power and utility and to estimate the incidence of single source clusters among human listeriosis cases. A scan statistic with an underlying Poisson distribution was used to detect the occurrence of temporal clusters caused by indistinguishable subtypes. A space-time scan statistic was used to evaluate spatial and temporal clustering on the basis of county of patient residence and a 3-month window.

Materials and Methods

Isolates and Case Reporting

In New York State, Public Health Law 2102 requires that laboratories and physicians immediately report isolation of *L. monocytogenes* from a sterile site (e.g., blood or cerebrospinal fluid) to public health authorities (16).

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Furthermore, local diagnostic and clinical laboratories are asked to submit all *L. monocytogenes* isolates to the New York State Department of Health Wadsworth Center. Through this system, *L. monocytogenes* isolates from cases of human invasive disease among New York State residents (excluding New York City, which is served more directly by the local health department) were collected over 44 months (November 1996 through June 2000). Only one isolate per patient was analyzed; therefore, each isolate in this study represents a single, unique listeriosis case. All isolates were confirmed by conventional biochemical tests at the Wadsworth Center. Standardized *L. monocytogenes* serotyping reagents were not available and serotyping was thus not performed.

County health departments reported epidemiologic information to the New York State Department of Health's Bureau of Communicable Disease. Local health department's systematic review of case reports aided identification of potential outbreak cases when large increases in listeriosis cases (irrespective of subtype) were reported. Our study is a retrospective laboratory subtype analysis, which did not include routine comprehensive risk factor analysis (i.e., history of food eaten).

Automated Ribotyping

Ribotyping was performed by using the restriction enzyme *Eco*RI and the RiboPrinter Microbial Characterization System (Qualicon Inc., Wilmington, DE) as previously described (17,18).

PFGE Analysis

PFGE was performed according to PulseNet protocol (15). *Apa*I PFGE patterns typically display more bands than *Asc*I patterns and may offer higher levels of discrimination; however, *Asc*I patterns typically have patterns with bands that are more easily analyzed by software and the human eye because of greater average distances between bands. While the current PulseNet protocol (15) recommends the use of both *Apa*I and *Asc*I for PFGE typing of *L. monocytogenes,* only *Asc*I was used in this study, which was initiated before formal inclusion of *L. monocytogenes* into PulseNet. Bacterial cultures were embedded in agarose, lysed, washed, and digested with the restriction enzyme *Asc*I for 4 h at 37°C and electrophoresed on a Chef Mapper XA (BioRad Laboratories, Hercules, CA) at 6 V/cm for 22 h with switch times of 4 s to 40.01 s. Pattern images were acquired by using a BioRad Gel Doc with Multi Analyst software (Bio-Rad Laboratories) (v. 1.1) and compared by using the Applied Maths Bionumerics (Applied Maths, Saint-Martins-Latem, Belgium) (v. 2.5) software package. Pattern clustering was performed by using the unweighted pairs group matching algorithm and the Dice correlation coefficient (15).

Strain Nomenclature

Ribotype patterns were automatically assigned a DuPont ID (e.g., DUP-1044) by the Riboprinter Microbial Characterization System (Qualicon, Inc.); each pattern was confirmed by visual inspection. If visual inspection found that a given DuPont ID included more than one distinct ribotype pattern, each pattern was designated by an alphabetically assigned additional letter (e.g., DUP-1044A and DUP-1044B represent two distinct ribotype patterns within DuPont ID DUP-1044). Distinct ribotype patterns within a given DuPont ID generally differed by position of a single weak band. If a ribotype pattern did not match a DuPont ID pattern with a similarity >0.85, a type designation was assigned manually based on the ribogroup assigned by the instrument (e.g., ribogroup 116-363-S-2). Ribotype patterns (and other subtype data) for isolates in this study are available for comparison on the Internet (available from: URL: www.pathogentracker.net). PFGE patterns differing by at least one band from a previously recognized type were given an indexed type comprising a two-letter geographic prefix, a four-digit year of first isolation, a three-letter restriction enzyme code, and a four-digit sequential number (e.g., NY1996ASC0001).

Simpson's Index of Discrimination

The suitability of typing methods for differentiation of strains was determined by using Simpson's Numerical Index (19). This index was calculated for each typing method, as well as for the combination of both methods.

Cluster Detection Algorithm by Using a Scan Statistic

The scan statistic (20,21) maintains the assumption that an underlying Poisson distribution and a stable population at risk over time describes the occurrence of rare events. This statistic tests the null hypothesis that the incidence of events within a given time window is equal to the incidence of events outside the window. We used a conditional Poisson distribution to describe the occurrence of individual *L. monocytogenes* subtypes over 44 months. Since the incubation period of listeriosis can be up to 70 days, we determined the temporal distribution of ribotypes and PFGE types for both 1- and 3-month windows. To determine the threshold value of occurrences, indicating a larger than expected number of events per window, we compared the number of occurrences in a given period to the expected maximum number of events in a given window. The expected number is calculated under the assumption that individual occurrences occur randomly with an identical rate over time. The conditional Poisson probability is given by: $P(n \mid N, p) = Pr(n_{obs} \geq n)$, where n_{obs} is the observed cluster size in a window, N is the total number of events during the total period, and p is the relative window length (the length of the window in months divided by the

length of the total period). Statistically significant clusters were identified when the number of isolates with a given subtype in the study window was greater than expected under the Poisson assumption. Exact p values were obtained from statistical tables (22). Clusters of ribotypes or PFGE types were evaluated by using a Poisson distribution with a mean rate equal to the number of occurrences divided by the total observation period. No corrections for seasonality were applied because analysis was performed at the subtype level and a large number of subtypes were observed. Furthermore, the prevalence of the different subtypes did not vary consistently by season, and correction was not warranted. Time-space clustering was evaluated by using a similar algorithm (SatScan 2.1.3, National Cancer Institute 1998) (23); however, only 3-month windows were evaluated. Case numbers were converted to case rate (cases/100,000) to account for the source population size. Time clustering within a 3-month window was combined with space clustering in an area with a maximum space window size of 15% of the total space. Kulldorff (24) recommended using a maximum space window size of up to 50% of the total space, while others reported using smaller values on the basis of the geographic boundaries studied. Norstrom et al. (25) used a 10% space window to avoid scanning outside the geographic region of study. We used a window of 15% of the total space. Since exact patient location (zip code or address) was not available, analysis was performed by using county of residence data, which provided a better chance of identifying local clusters within a small food distribution area. Each case was assigned the spatial coordinates of the county in which the patient was residing (Figure 1). Statistical significance for all tests was defined as $p \leq 0.05$.

Results

Cases

From November 1996 through June 2000, a total of 135 *L. monocytogenes* isolates were collected from human cases, with four mother/newborn pairs of isolates. All four isolates from the newborns matched the subtype of the respective mother and were not included in our analysis. The incidence of reported isolates ranged from 0 to 13 per month, with a median of three. Isolates were seasonally distributed with peaks in July 1997 (n=5), October 1998 (n=13), and September 1999 (n=9). On the basis of the 2000 census population estimate of 10,968,179 (26) for New York State (excluding New York City), we detected a listeriosis rate of 0.33 cases per 100,000. Cases were distributed across the state (Figure 1). Case-patient ages ranged from <1 day to 98 years with a median age of 66 years. Gender was reported for all cases; 76 (58%) of case-patients were female.

A total of 34 ribotypes and 74 PFGE types were differentiated among the 131 human isolates; 19 ribotypes and 50 PFGE types were unique (i.e., represented by only one patient isolate). Ribotypes DUP-1044A and DUP-1052A were each prevalent in >10% of cases, and these two ribotypes alone accounted for 39% of cases. One PFGE type (NY1997ASC0010) accounted for 13% of cases. No other PFGE types accounted for >5% of cases.

Discriminatory Ability of Typing Methods

Simpson's Index was used to determine the discriminatory power of the subtyping methods used. The D value was 0.923 for ribotyping, 0.975 for PFGE, and 0.980 for combined use of both typing techniques. PFGE further discriminated most ribotypes (Table 1); however, three iso-

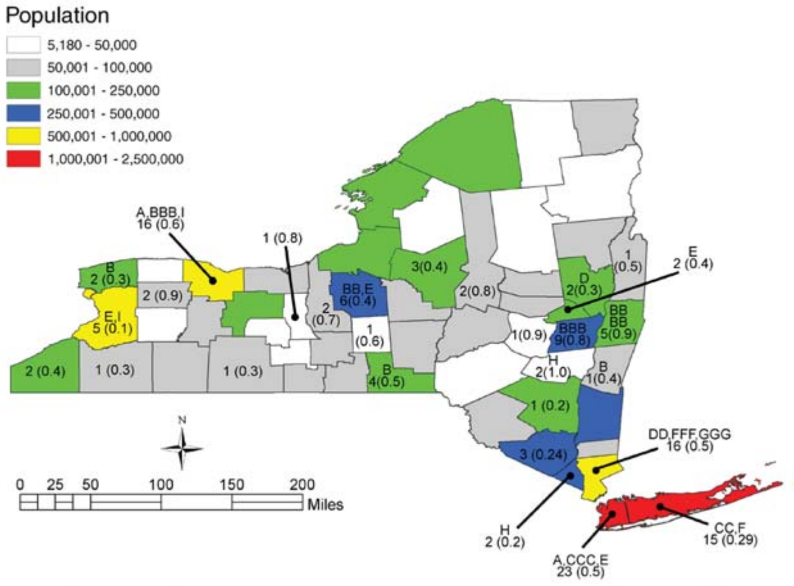

Figure 1. Dispersion of listeriosis cases, New York State (excluding New York City), November 1996–June 2000. Comparison of New York State population base overlaid with temporal listeriosis clusters from Table 1 (indicated by letter; defined by ribotype and pulsed-field gel electrophoresis type). Cases per county and annualized rate per 100,000 (in parentheses) are shown. New York City listeriosis data are not included in this study.

Table 1. Temporal clusters of human listeriosis identified by ribotyping, pulsed-field gel electrophoresis (PFGE), or both by using a 3-month window, New York State, November 1996–June 2000[a]

Cluster	Ribotype	p values for temporal scan statistic with: Ribotype	PFGE type	Date of specimen collection	*Asc*I PFGE type (no.)	PFGE relatedness
A	DUP-1044A	NS	≤0.05	**Sep 1997**	**NY1997ASC0016**	**Indistinguishable**
				Sep 1997	**NY1997ASC0016**	**Indistinguishable**
B	DUP-1044A[a]	≤0.01	≤0.05	**Sep 1998**	**NY1997ASC0006**	**1 band from NY1997ASC0010**
				Oct 1998	**NY1997ASC0010 (7)**	**Indistinguishable**
				Nov 1998	**NY1997ASC0010 (2)**	**Indistinguishable**
				Dec 1998	**NY1997ASC0010 (4)**	**Indistinguishable**
C	DUP-1042B	≤0.05	N/A	Dec 1998	NY1999ASC0045	>5 bands from all others in cluster
				Feb 1999	**NY1997ASC0017**	**2 bands from NY1997ASC0014**
				Feb 1999	NY1996ASC0001	>5 bands from all others in cluster
				Feb 1999	NY1999ASC0050	>5 bands from all others in cluster
				Mar 1999	**NY1997ASC0014**	**2 bands from NY1997ASC0017**
D	DUP-1042B	NS	≤0.05	Aug 1999	NY1997ASC0017	>5 bands from all others in cluster
				Aug 1999	**NY1999ASC0050**	**2 bands from NY1999ASC0061**
				Aug 1999	**NY1999ASC0061**	**Indistinguishable**
				Sep 1999	**NY1999ASC0061**	**Indistinguishable**
E	116-363-S-2	≤0.01	≤0.01	**Aug 1999**	**NY1999ASC0052**	**Indistinguishable**
	116-363-S-2	≤0.01	≤0.01	**Sep 1999**	**NY1999ASC0052**	**Indistinguishable**
	DUP-1044B	NS	≤0.01	**Sep 1999**	**NY1999ASC0052**	**Indistinguishable**
	116-363-S-2	≤0.01	NS	Sep 1999	NY1999ASC0064	4 bands from NY1999ASC0052
F	DUP-1053A	≤0.05	≤0.05	**Sep 1999**	**NY1999ASC0069**	**Indistinguishable**
				Nov 1999	**NY1999ASC0069 (2)**	**Indistinguishable**
				Dec 1999	**NY1999ASC0069**	**Indistinguishable**
G	DUP-1052A[a]	NS	≤0.05	**Oct 1999**	**NY1997ASC0018 (3)**	**Indistinguishable**
H	DUP-1043	≤0.01	≤0.05	**Jan 2000**	**NY2000ASC0075**	**Indistinguishable**
				Feb 2000	**NY2000ASC0075**	**Indistinguishable**
I	DUP-1045B	≤0.05	≤0.05	**Apr 2000**	**NY2000ASC0077**	**Indistinguishable**
				May 2000	**NY2000ASC0077**	**Indistinguishable**
				May 2000	NY2000ASC0083	>5 bands from NY2000ASC0077

[a]Epidemiologically linked clusters; cluster B linked to eating hot-dog brand 1, cluster G to eating paté brand 2; bold, isolates for which PFGE patterns were supportive of respective clusters; NS, not significant (p>0.05); N/A, the statistical significance of occurrence of a unique PFGE type cannot be tested.

lates with indistinguishable PFGE types were further differentiated into two ribotypes (Table 1, cluster E).

Cluster Detection

Ribotyping and PFGE subtyping data were analyzed separately by using a scan statistic on 1- and 3-month windows to detect statistically significant clusters of identical ribotypes and PFGE types. A total of 9 clusters representing 41 (31%) cases were detected by ribotyping, PFGE, or both (Tables 1 and 2). Clusters were detected throughout the study period (Figure 2). Two clusters (B and G) were epidemiologically linked to national outbreaks and known sources and included 17 (13%) cases. The remaining seven clusters were not epidemiologically defined as outbreaks, and the exact source of exposure was undetermined. Ribotype-based scanning with 1-month windows detected two clusters (Table 1, B and E), while scanning with 3-month windows detected six clusters (B,C,E,F,H, and I). PFGE-based scanning with 1-month windows detected five clusters (A,B,E,F,G), while scanning with 3-month windows detected eight clusters (A,B,D,E,F,G,H, and I). All clusters identified by using 1-month windows were also identified by using 3-month windows.

A total of six ribotype-based clusters (Table 1; B, C, E, F, H, and I) of two or more isolates ($p \leq 0.05$) were detected, representing a total of 31 (24%) cases. PFGE alone identified eight clusters (A, B, D, E, F, G, H, and I), representing a total of 31 (24%) cases. Ribotyping and PFGE results were used to further refine clusters detected by the scan statistic. All six ribotype clusters contained at least two indistinguishable or closely related (≤3 bands different) PFGE patterns. For the purpose of refining ribotype clusters, we interpreted PFGE patterns differing by ≤3 bands from each other as possibly being clonally related and sharing a recent enough common ancestor to be grouped together for epidemiologic investigations (27). Three of these clusters (C, E, and I) contained one or more isolates removed from the ribotyped-based cluster because

Table 2. Comparison of statistically significant temporal listeriosis clusters stratified by subtyping technique used to detect and confirm each cluster

Clusters	Cluster definition	No. of clusters	No. of cases (%)[a]
1. Clusters detected by ribotype or PFGE	Ribotype clusters or PFGE clusters detected by using the scan statistic ($p \leq 0.05$)	9	41 (31)
2. Ribotype clusters	Indistinguishable ribotype pattern clusters detected by the scan statistic ($p \leq 0.05$)	6	31 (24)
2a. Ribotype clusters supported by PFGE	Ribotype clusters, containing closely related PFGE types (≤3 bands difference)	6	26 (20)
3. PFGE clusters	Indistinguishable PFGE patterns detected by the scan statistic ($p \leq 0.05$)	8	31 (24)
3a. PFGE clusters supported by ribotype	PFGE clusters, which contained identical ribotype patterns	8	30 (23)
4. Clusters supported by ribotype and PFGE	Clusters detected as 2a and 3a	5	23 (18)
5. Epidemiologically linked ribotype or PFGE clusters	Clusters detected by ribotype, PFGE, or both and supported by epidemiologic data	2	17 (13)

[a]Based on total sample population of 131 isolates; PFGE, pulsed-field gel electrophoresis.

they were considered not closely related to the most common PFGE pattern in the respective cluster (see Figure 3 for two examples of ribotype clusters with multiple PFGE types and two examples of ribotype clusters with indistinguishable or closely related PFGE types). Overall, ribotype clusters that were further supported by indistinguishable or closely related PFGE types represented 26 (20%) cases (Table 2). Of the eight PFGE clusters detected, all, except one (Figure 3, cluster E), comprised isolates with identical ribotypes. Overall, five clusters (B, E, F, H, and I; 23 cases) were detected by the temporal scan statistic on the basis of both ribotype and PFGE data.

Space-time cluster analysis independently identified three of the ribotype clusters (B, G, and H) and five of the PFGE clusters (B, D, G, H, and I). While some geographic clusters were located within one county (D and G), others comprised cases in one or more counties (B, H, and I). Cluster B comprised two main geographic clusters; one included five cases (Rensselaer and Columbia Counties), and the other included six cases (Broome, Monroe, and Onondaga Counties). An additional three cases from cluster B (Table 1), detected by the temporal scan statistic, were not detected by the space-time analysis (two cases in Albany and one in Erie Counties).

Discussion

L. monocytogenes causes a rare, severe human foodborne disease and is responsible for an estimated 2,500 human cases and 500 deaths annually in the United States (28). Most of these cases have been considered sporadic, and comparatively few outbreaks have been reported worldwide (1). The best quantitative estimates of the true number of *L. monocytogenes* infections come from the FoodNet program, which conducts population-based active laboratory surveillance for foodborne diseases at 10 sites in the United States that represent 10.8% of the U.S. population (2). FoodNet aggregate data from 1996 to 2000 show that the reported listeriosis rates among participating sites ranged from 3 to 6 cases per million population per year. The 131 case isolates collected over 44 months in this study translate to a rate of 3.3 cases per million population per year. This rate is within the reported case prevalence from FoodNet sites (2), indicating that the capture rate achieved in this study is within the expected range.

Comparison of Genotyping Methods

Rapid, reproducible, and discriminatory subtyping methods are important in conducting effective surveillance. While others have shown that PFGE and ribotyping are highly discriminatory for typing *L. monocytogenes* (29–32), no comprehensive reports evaluated typing strategies on human isolates from a broad-based surveillance program (29–32). While our data show that *Eco*RI ribotyping and *Asc*I PFGE typing provide discriminatory subtyping approaches for human listeriosis isolates (Simpson's index of 0.923 and 0.975, respectively), we also found that most ribotypes could be further differentiated by *Asc*I PFGE. Since PFGE types of epidemiologically related isolates may differ by ≤3 bands from each other (27), small clusters (e.g., cluster D) involving related but distinct (≤3 bands difference) isolates may not be detected by PFGE typing but can be detected by ribotyping, which appears to target more conserved genetic characteristics.

Detection and Definition of Listeriosis Clusters and Outbreaks

Within the 3 1/2-year study period covered by this report, nine putative case clusters (representing 31% of cases) were identified by using the scan statistic based on ribotyping or PFGE data. Application of the scan statistic for cluster detection ensured that putative clusters accounted for the relative abundance of *L. monocytogenes* ribotypes and PFGE types. Five clusters, representing 18% of all cases, were supported statistically by both subtyping

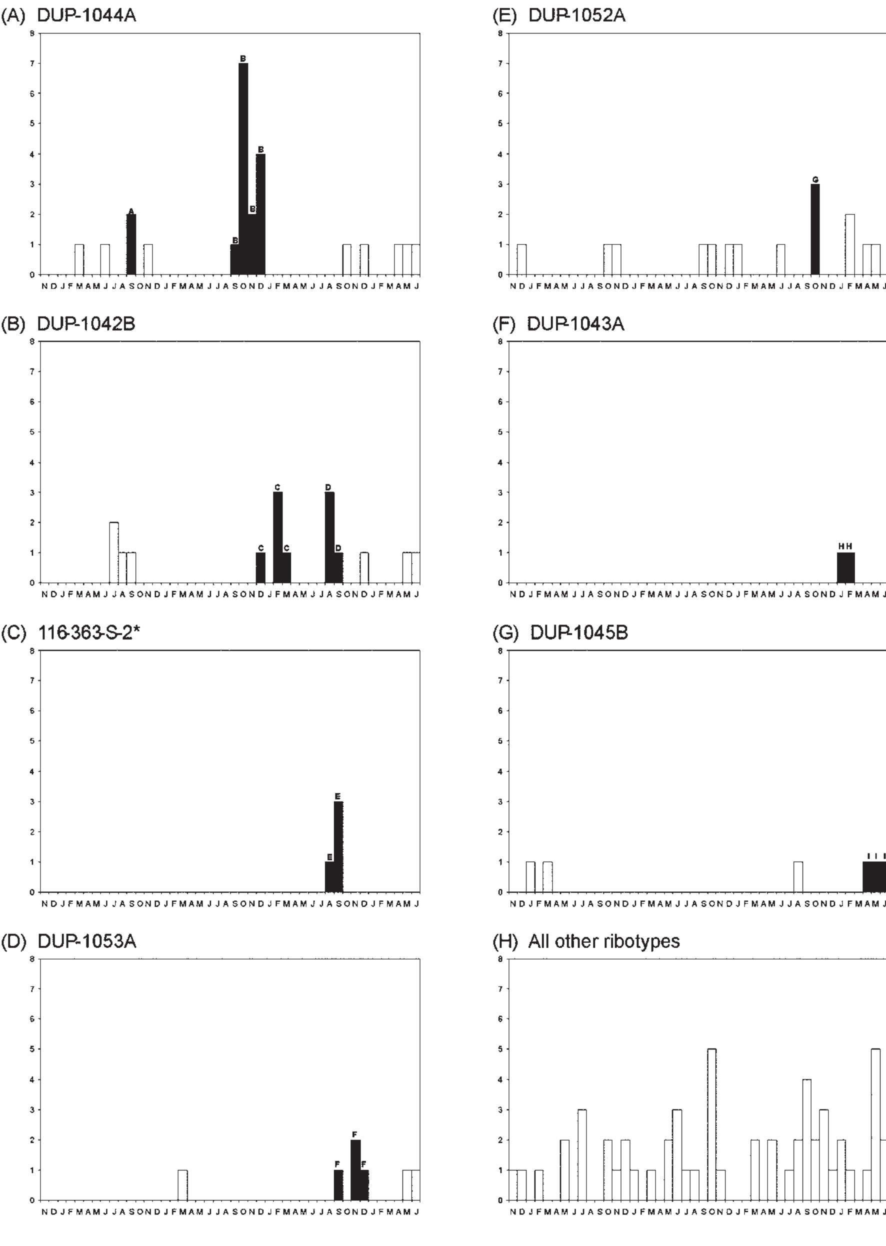

Figure 2. Temporal distribution of listeriosis clusters detected based on ribotype or pulsed-field gel electrophoresis (PFGE) data, using a 3-month window scan statistic. Panels A–G each show the distribution of cases caused by a specific ribotype; ribotypes are denoted in the header of each panel. For panel C, one case caused by ribotype DUP-1044B is included with cases caused by ribotype 116-363-S-2 based on a PFGE match (Table 1, cluster E). Cases, which are part of statistically significant ribotype or PFGE clusters, are denoted by dark bars and labeled by cluster designation (A–I, see Table 1). Open bars indicate cases that were not part of a cluster detected by the scan statistics. Panel H shows human cases, which did not represent clusters and were not caused by any of the ribotypes shown in panels A–G. The X-axis of each panel represents November 1996 to June 2000.

methods. Of the six ribotype clusters identified by using the scan statistic, all contained isolates with closely related PFGE types. When refined to include only closely related PFGE types (≤3 bands difference), these six clusters represent 20% of the cases reported during the surveillance period. Cluster C contained five PFGE types, including three that were more than five bands different and two that were two bands different, indicating that these cases were unrelated. The relevance of both PFGE and ribotyping-based cluster detection by means of the scan statistic is supported by the observation that two of the clusters detected by one or both methods represent clusters that were part of epidemiologically confirmed multistate human foodborne listeriosis outbreaks. Cluster B (Table 1), which included 14 cases in New York State that were ribotype DUP-1044A, was part of a multistate outbreak with 101 cases (including 21 deaths) linked to eating *L. monocytogenes*–contaminated hot dogs (12,33). All cases from Cluster B, including the one case with a PFGE pattern that differed by a single band from the other isolates (Figure 3), were epidemiologically linked to the national outbreak. A second cluster (cluster G) was connected to cases in Maryland, New York, and Connecticut and was linked by subtyping and epidemiologically to contaminated paté (34,35). These two clusters represented 13% of all cases reported in New York State during this

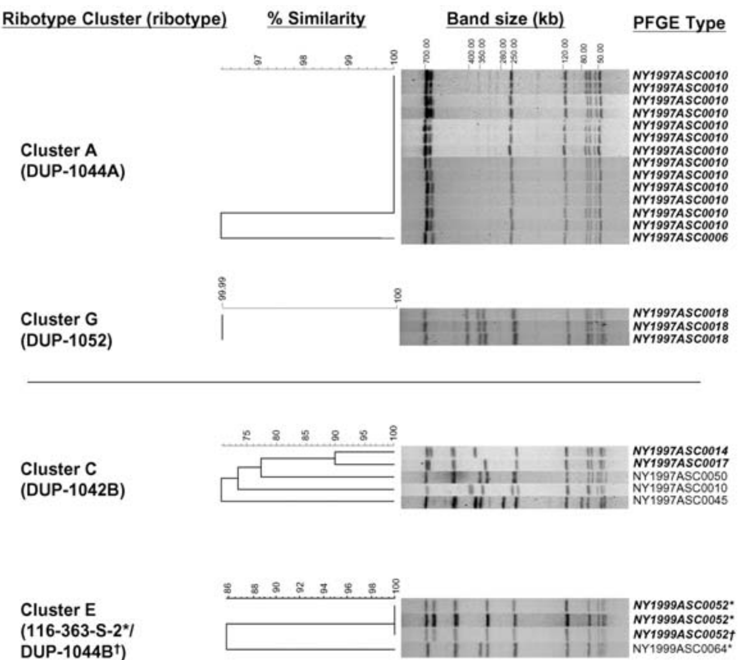

Figure 3. Comparison of *AscI* pulsed-field gel electrophoresis (PFGE) patterns for isolates from selected ribotype clusters. AscI PFGE types are shown for two clusters representing epidemiologically confirmed outbreaks (A and G), one ribotype cluster that was further discriminated by PFGE typing (C), and one cluster with overlapping PFGE and ribotype clusters (E). Isolates with <3 bands difference are shown in bold. The percent similarity does not reflect true phylogenetic distance.

surveillance period. Because of the retrospective nature of this study, no epidemiologic data were available to link the cases representing the other subtype clusters.

While many reports claim that most listeriosis cases are sporadic (2–4,7), our data show that a considerable proportion of human listeriosis cases represent subtype clusters, some or all of which may represent common source outbreaks. Such clusters may have also occurred before 1997 and in other states and countries. While many of the subtype clusters detected in New York State appear to be small, some involved additional cases outside the state, and some cases connected with these clusters may never have been diagnosed. As nationwide surveillance and genotyping systems such as PulseNet (36) become fully implemented, a much larger number of human listeriosis clusters and outbreaks may be recognized and linked to specific food sources. Subtyping methods will only provide their full public health benefit if routine food histories are obtained for all listeriosis patients to provide the epidemiologic support for putative single genotype clusters. Complete routine food histories were not obtained as part of this study but were administered when putative outbreaks (such as clusters B and G) were detected before application of the statistical algorithm described here.

While some clusters defined by the temporal scan statistic also represented statistically significant spatial clusters (Figure 1, D, G, H, and I;), other temporal clusters included cases distributed across the state (A, B, C, E, and F). Cluster B included two smaller space-time clusters as well as other cases distributed across the state; all of these cases were epidemiologically linked. These patterns are consistent with those of previously reported human listeriosis outbreaks. Some previous outbreaks of listeriosis have been represented as geographic clusters associated with localized consumption of a contaminated food item (e.g., outbreaks in North Carolina [37] and California [38] linked to Hispanic-style cheeses). Other outbreaks were geographically dispersed and included cases in many states; these clusters were caused by a widely distributed contaminated food item (such as the multistate outbreak in 1998–99 [12]; cluster B). Our results further suggest that human listeriosis clusters and outbreaks may occur in two distinct patterns, including localized, geographically confined, and dispersed clusters. This epidemiologic spreading pattern indicates that time clustering is probably at least as effective in detecting clusters as combined space-time clustering.

Cluster Detection Methods

While some efforts to track *L. monocytogenes* subtypes responsible for human cases over time have been published (39,40), we show that the use of comprehensive multimethod genotyping approaches in conjunction with formal statistical means for detecting putative listeriosis clusters may help provide a better understanding of the epidemiologic characteristics of this disease. While the combination of typing and normal distribution-based statistical algorithms for outbreak detection has been shown to be effective for detecting outbreaks for more common foodborne diseases such as salmonellosis (41), different approaches are needed to effectively detect clusters for rare diseases such as listeriosis. Therefore, we used PFGE

and ribotyping subtyping in conjunction with the Poisson-distribution-based scan statistic to detect listeriosis clusters. The scan statistic was chosen since this method has previously been applied to detect clusters of other rare diseases, e.g., variant Creutzfeldt-Jakob disease (24,42–43). Because of the long incubation period of listeriosis, the scan statistic was performed by using both 1- and 3-month windows. Our data showed that all clusters detected with the 1-month window were also detected with the 3-month window size. Further validation of appropriate window sizes for these analyses by using epidemiologically confirmed outbreaks will be necessary to define the optimal parameters for the scan statistic analysis. While *Eco*RI ribotyping was shown to be less discriminatory than *Asc*I PFGE typing, PFGE patterns differing by $\leq$3 bands from each other may possibly be clonally related and share a recent enough common ancestor to be grouped together for epidemiologic investigations (27). Consequently, the use of the more discriminatory PFGE subtyping data alone may sometime miss clusters caused by clonally related isolates, which may not necessarily share completely identical PFGE patterns, if only the completely identical PFGE patterns (0 band difference) are grouped together as a single PFGE type. The use of only ribotyping data may overestimate the number of clusters because of the lower discriminatory ability of ribotyping. We showed that PFGE data further refined the initially defined ribotype clusters and eliminated clusters that contained isolates with distinct PFGE subtypes.

Conclusion

Conventional surveillance for listeriosis and other foodborne diseases often relies upon species or serotype characterization to define reportable conditions, yet for many organisms genotyping can provide improved discrimination below the species or serotype level. In conjunction with statistical analyses, routine genotyping allowed us to identify a considerable number of putative temporal clusters of listeriosis. Our data show that 13% of reported human listeriosis cases in New York State represented epidemiologically supported single-source, multicase clusters. On the basis of molecular subtyping data alone, as many as 31% of the listeriosis cases may have represented clusters. We propose that a considerable number of human listeriosis cases may occur in clusters, many or some of which may represent single-source outbreaks that in the past went undetected. The combined use of molecular subtyping methods, statistical data analysis, and epidemiologic investigations thus may further improve our ability to detect human listeriosis outbreaks.

The U.S. Department of Health and Human Services Healthy People 2010 plan calls for a reduction of human listeriosis from 0.5 to 0.25 cases per 100,000 by the year 2010 (44). Efforts to reduce *Listeria* species in the processing environment appear to have reduced the incidence of listeriosis from a peak of 0.8 cases per 100,000 in the early 1990s, but the incidence has remained at approximately 0.3–0.6 cases per 100,000 since 1996 (7,45). Our study suggests that single-source clusters represent a much larger number of listeriosis cases than previously assumed. We provide a model for an integrated, statistically based, molecular subtyping approach to identifying putative foodborne listeriosis clusters. In conjunction with broad-based collection of conventional epidemiologic data, this approach may allow for more rapid detection of even smaller outbreaks, which currently are often unrecognized. Rapid cluster detection can help detect and eliminate outbreak sources and prevent additional cases, thus providing an opportunity to reduce the overall incidence of foodborne listeriosis. Improved outbreak detection furthermore will provide an opportunity to better define the specific food sources of human listeriosis cases.

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Mr. Sauders is a Ph.D. student in the Department of Food Science, Cornell University, and a former Centers for Disease Control and Prevention/Association of Public Health Laboratories Emerging Infectious Diseases Training Fellow. His research interests focus on using molecular subtyping strategies to better understand the ecologic, epidemiologic, and transmission dynamics of foodborne infectious diseases.

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Bioterrorism-related Inhalational Anthrax in an Elderly Woman, Connecticut, 2001

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On November 20, 2001, inhalational anthrax was confirmed in an elderly woman from rural Connecticut. To determine her exposure source, we conducted an extensive epidemiologic, environmental, and laboratory investigation. Molecular subtyping showed that her isolate was indistinguishable from isolates associated with intentionally contaminated letters. No samples from her home or community yielded *Bacillus anthracis*, and she received no first-class letters from facilities known to have processed intentionally contaminated letters. Environmental sampling in the regional Connecticut postal facility yielded *B. anthracis* spores from 4 (31%) of 13 sorting machines. One extensively contaminated machine primarily processes bulk mail. A second machine that does final sorting of bulk mail for her zip code yielded *B. anthracis* on the column of bins for her carrier route. The evidence suggests she was exposed through a cross-contaminated bulk mail letter. Such cross-contamination of letters and postal facilities has implications for managing the response to future *B. anthracis*–contaminated mailings.

On November 19, 2001, a suspected case of inhalational anthrax in a 94-year-old woman was reported to the Connecticut Department of Public Health (CTDPH) (1–3). This was the first case of *Bacillus anthracis* infection reported to the CTDPH since 1968 and the eleventh inhalational anthrax case in the United States since October 4, 2001 (1–6). The patient's symptoms of fever, fatigue, malaise, dry cough, and shortness of breath began 20 days after the last confirmed inhalational anthrax patient became ill and 36 days after the last known intentionally contaminated letters, addressed to U.S. Senators Thomas Daschle and Patrick Leahy, were postmarked in Trenton, New Jersey (1–4) (Figure 1).The patient in Connecticut was not in the known categories of intentionally contaminated letter recipients and was not a postal worker or a mailhandler (1,5). This report describes the epidemiologic and environmental investigation conducted to determine whether her case was related to the other bioterrorism-related cases; whether she was the only case in Connecticut or a sentinel of a larger outbreak; and the source, place, and time of her exposure. The clinical aspects of the case have been described (2,3).

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Methods

Isolate Comparison

A subculture of the patient's *B. anthracis* blood culture isolate was examined for species confirmation, antibiotic susceptibility testing, and molecular subtyping by multiple-locus variable-number tandem repeat analysis, which examines eight loci on the *B. anthracis* genome (7,8). The isolate was compared with previous bioterrorism-related isolates on the basis of antibiotic susceptibilities and molecular subtyping.

Surveillance

We conducted retrospective surveillance for additional cases of human or animal anthrax in Connecticut for September 1 to November 30, 2001, by using data from death certificates; medical examiner, laboratory, and postal worker absentee records; and surveys of licensed veterinarians. We conducted prospective surveillance for additional cases of human or animal anthrax in Connecticut from November 20 to December 21, 2001, by using reports from hospital admissions, laboratories, healthcare providers, veterinarians, and animal control officers, and also reports from the U.S. Postal Service (USPS) on employee absenteeism (9,10).

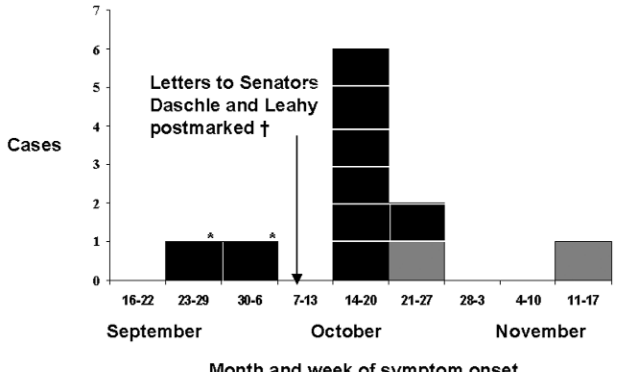

Figure 1. Bioterrorism-related inhalational anthrax cases by week of symptom onset—United States, 2001.The first two cases of inhalational anthrax occurred in Florida. Though no direct exposure source was found, environmental samples of the media company in which these two patients worked and the postal facilities serving the media company yielded *Bacillus anthracis* spores specifically implicating a *B. anthracis*–containing letter or package (4): †, the letters to Senators Thomas Daschle and Patraick Leahy were postmarked in the Trenton, New Jersey, processing and distribution center on October 9, 2001; ■ indicates a case of inhalational anthrax in a person with direct exposure to a *B. anthracis*–containing letter; ■ indicates a case of inhalational anthrax in a person with no known *B. anthracis* exposure.

Patient Epidemiologic Investigation

In collaboration with local, state, and federal law enforcement agencies, we identified the patient's activities, home visitors, and all places she visited in the 60 days preceding her symptom onset using her personal calendar and interviewing her family, friends, neighbors, physicians, and persons who cleaned her home. We also met with investigators of the 10th inhalational anthrax case from New York City to assess similarities between the two cases.

Patient Environmental Investigation

In the patient's home, environmental samples and selected personal effects were collected for culture during eight inspections conducted from November 20 to December 4. We obtained swab and wipe samples from clean, nonporous surfaces and vacuum samples from large or dusty nonporous or porous surfaces (11). Surface swab samples were collected by using synthetic swabs moistened with sterile saline or sterile water to sample such surfaces as vents; furniture; appliances, including vacuum cleaners; areas with dust; electrostatically charged surfaces, including a television screen; aerosolizing and misting devices, including an inhaler and a perfume bottle; and all places in the home where she might have handled her mail. Vacuum samples were collected by using high-efficiency particulate air (HEPA) vacuum cleaners equipped with a filter collection device to vacuum carpets, furniture, and clothing. Final intensified sampling was performed by using blowers to aerosolize particles throughout the living space, followed by air sampling that used high-volume air filtration devices and the placement of blood agar settle plates throughout the home. Personal effects collected from the home included pieces of mail, file folders, pieces of paper, used tissues, letter openers, pill bottles, an inhaler, photographs, and a calendar. Bulk samples of contents from the bags of vacuum cleaners normally used to clean the home were collected for culture. Nasal swabs were taken from all persons who spent >60 minutes in the patient's home during the 60 days before onset of her symptoms.

Outside the home, environmental samples were collected for culture from November 20 to December 2. We collected moist swab and vacuum samples from all indoor air spaces she had visited in the 60 days preceding her symptom onset. Moist swab samples were also collected at selected outdoor locations, including her mailbox and the soil around it, mailboxes on her street, soil in her yard, and soil at a local establishment rumored to be located on the site of a previous farm that was closed because of an anthrax outbreak among cattle during the early 1900s.

Postal Epidemiologic Investigation

USPS has a mail tracing system by which data are recorded from bar codes applied to letters in postal processing and distribution centers (PDCs). For first-class letters, canceling machines apply both an identification code (orange bar code on the back of the envelope) and a postnet code (black bar code on the front of the envelope) to envelopes. Bulk letters are not processed on canceling machines because they are presorted with the postnet code preapplied. When the identification code is used, canceling machines record the day of the month, the time of day, and the sequence in which each first-class letter was processed during a given half-hour interval. Sorting machines then use the postnet code to group both first-class and bulk letters that go to a particular address. Sorting machines record the total count of all letters processed on each sorting machine, but only first-class letters with identification codes include time of day and identity of the particular sorting machine. Consequently, identifying information on bulk letters is not recorded in the mail tracing system. All letters are processed on sorting machines approximately two to five times, progressing from an initial sort, to the five-digit zip code, through a specific final sort in which letters are sorted to collecting bins in the delivery sequence for the given postal carrier route (12).

We searched the patient's home for letters she received since September 1, 2001, and recovered letters from her office, personal files, and trash bins. The recovered letters were categorized as first-class or bulk and submitted to the

CTDPH laboratory for culture. First-class letters were checked against the USPS database for date and location of cancelation. To determine whether the patient received first-class letters from any PDCs that processed an intentionally contaminated letter (i.e., Trenton, NJ; Brentwood, Washington, DC; Morgan, New York City; and West Palm Beach, FL), we examined USPS data of outgoing mail from these facilities for first-class letters with Connecticut destination addresses. Data were reviewed from October 9 until either the date the particular PDC closed because of *B. anthracis* contamination or, if the PDC did not close, the date the patient's symptoms began (November 13). We chose October 9 as a starting date because the only recovered intentionally contaminated letters that resulted in cases of inhalational anthrax were postmarked on this date and because 36 days had already elapsed between this date and the onset of the patient's symptoms. Information on the date, time, and machines involved in processing these first-class letters was retrieved.

To identify all first-class letters the patient received, regardless of point of origin, we examined USPS data for first-class letters sorted in the Southern Connecticut PDC that served her local post office from October 9 to November 13. We retrieved available information on the date, time, and identity of a letter's originating PDC; however, information on the machines that processed a particular letter in the originating PDC is not available. To check for bulk letters sent to the patient's zip code from October 9 to October 16, 2001, we contacted mailing companies that used the Trenton PDC.

Postal Environmental Investigation

Environmental samples were collected at the Southern Connecticut PDC on five occasions. On November 11, 2001, 2 days before the patient's symptoms began, an independent contractor took surface samples from various locations with dry synthetic swabs as part of a nationwide USPS effort to identify contamination of selected PDCs. On November 21, a second independent contractor hired by USPS collected additional dry swab samples of surfaces, including 29 letter-canceling and -sorting machines, 4 flat- (magazine) sorting, and 4 parcel-sorting machines; air-handling units; and vacuum cleaner filters from different facilities. On November 25, we inspected and repeated sampling of similar locations using moist synthetic swabs. On November 28, guided by findings from epidemiologic investigations at the Brentwood PDC, we collected vacuum and moist synthetic 2x2-inch surface wipe samples from all letter-canceling and -sorting machines (13). After samples from three letter-sorting machines were positive for *B. anthracis,* we collected additional moist surface wipe samples from each column of collecting bins on the three letter-sorting machines with samples yielding *B. anthracis* and from the sorting machine that completed the final sort of letters that included the patient's mail carrier route. We also collected nasal swabs for culture from employees of the Southern Connecticut PDC during November 21 to 24.

At the patient's local post office, we obtained dry and moist synthetic surface swab and vacuum samples of the mail-sorting area, computer screens, gurneys, carts, loading dock, and vehicle serving her postal carrier route on four occasions during November 21 to December 2, 2001. We collected nasal swabs for culture from employees of her local post office during November 21 to November 24.

Postal Laboratory Studies

All environmental samples were tested either in the CTDPH laboratory or, when it had reached its capacity, a contract laboratory in Texas. Surface swab, nasal swab, and vacuum samples and blood agar settle plates were analyzed in the CTDPH laboratory. Surface wipe, vacuum, and air-filter samples, and bulk contents of vacuum cleaners were analyzed in the contract laboratory in Texas. All swab specimens were plated directly onto sheep blood agar and handled using standard procedures (14). Vacuum, air-filter, and surface wipe samples, as well as vacuum cleaner bag contents, were processed with the following extraction procedure: the specimen contents were placed into a sample-processing solution and centrifuged to create a pellet; the pellet was resuspended in 0.3% Tween 20 in phosphate-buffered saline; the resuspended solution was then heat-shocked; and one tenth of the resuspended solution was placed on sheep blood plates (14). All suspicious colonies were screened by Gram stain and motility testing and confirmed by gamma phage lysis and polymerase chain reaction (14). All samples from the patient's home that underwent the extraction procedure and tested negative were retested with one half of the remaining sample-processing solution.

Results

Isolate Comparison and Surveillance

Molecular subtyping results identified the isolate as being of MLVA genotype 62, and antibiotic susceptibilities of the isolate were indistinguishable from those of the other human anthrax patients confirmed nationwide since October 4, 2001 (4,8). Retrospective and prospective surveillance did not identify any additional human or animal anthrax-related illness (9,10).

Patient Epidemiologic Investigation

The patient lived in a central Connecticut town with a population of 9,821 persons (2). She lived alone in a ranch-

style home with a basement, located on a half-acre, partially wooded lot on a residential side street approximately one third of a mile from the state road. The home entryway was readily visible to homes across the street.

She spent most of her time inside her home reading or watching television and did not spend time outdoors. She did not shop or cook; family and friends provided food and household supplies. Her home was clean and well-organized. She had no hobbies requiring woolen items (e.g., knitting or crocheting), goat hair, or leather. A family member assisted her in paying bills, reading unfamiliar letters, and handling bank transactions. In the 60 days preceding symptom onset, her only visitors besides family and close friends were her church pastor, approximately 25 Halloween trick-or-treaters, and two persons from her cleaning service. No unusual visitors or solicitors were noted by friends or neighbors.

She did not drive; family and friends provided transportation. She was always accompanied when outside the home and limited her time away from home to <2 hours to prevent fatigue. Her activities outside her home consisted of weekly hair salon appointments, routine physician visits, lunch outings with friends, weekly church attendance, visiting another church for a Christmas fair, and voting at the town hall. No unusual persons or occurrences were noted by those who accompanied her during these outings.

When she was compared with the 10th case-patient with inhalational anthrax in New York City, limited similarities were found: both were women >60 years of age, lived alone in clean homes, wore hats, and had a bottle of the same brand of perfume (which was sampled for the presence of *B. anthracis*). They had no brand of medication, physicians, hobbies, social networks, or geographic area in common.

Patient Environmental Investigation

Cultures of all 258 samples and 84 personal effects from the patient's home were negative for *B. anthracis.* The 181 samples from the indoor air spaces she visited in the 60 days preceding symptom onset (including 11 restaurants, seven cars, five physician's offices, five homes of neighbors or close friends, two churches, a bank, a hair salon, and a public building) also cultured negative for *B. anthracis.* Cultures of the 17 samples from the selected outdoor locations and 16 nasal swabs from visitors to her home also were negative for *B. anthracis.*

Postal Epidemiologic Investigation

We recovered 29 letters from her home postmarked after September 1, 2001: 7 canceled first-class letters and 23 presorted bulk letters. These 29 letters likely did not represent the entire number of letters she had received since September 1. Of the six first-class letters, only one was postmarked after October 9 (October 26). All first-class letters were sliced open along the top border of the envelope, whereas bulk letters, mainly solicitations or credit-card offers recovered from her garbage, had been torn in half. Samples from all 29 recovered letters were negative for *B. anthracis*.

The Morgan and West Palm Beach PDCs did not save records of outgoing mail for the period of interest. The Trenton and Brentwood PDCs did not send first-class letters directly to her address during October 9 to October 21 (date after which both facilities were closed because of *B. anthracis* contamination). Five first-class letters from the Trenton PDC and three first-class letters from the Brentwood PDC were sent to her postal carrier route during this time. Two of the letters from the Trenton PDC were processed approximately 3 hours after the Daschle and Leahy letters and on the same canceling machine. None of these letters were delivered to an address on her street, none coincided with a first-class letter to the patient, and none were recovered.

USPS data from the Southern Connecticut PDC showed that eight first-class letters, only one of which was recovered, had been sent to her address during October 9 to November 13, 2001. All eight originated in Connecticut and were canceled at the Southern Connecticut PDC.

While examining the USPS data, we identified and recovered a first-class letter (letter A) that was sorted in the Trenton PDC 283 letters (approximately 15 s) after the intentionally contaminated letter to Senator Leahy. Letter A was processed in the Southern Connecticut PDC and the patient's local post office and delivered to an address approximately 4 miles away on a different mail carrier route. Three separate moist swab samples taken from the outside of letter A's envelope yielded 1, 3, and 7 CFU of *B. anthracis,* respectively. Cultures of moist swab samples from the inside of the envelope and its contents were negative for *B. anthracis*. The sorting machine in the Southern Connecticut PDC that first processed letter A could not read the postnet code. Therefore, letter A was removed from the automated system and hand-sorted. Eight moist wipe and vacuum cleaner samples from the sorting machine in the Southern Connecticut PDC that first sorted letter A were negative for *B. anthracis* by culture as were 36 moist swab and vacuum cleaner samples from the home that received letter A, including the box that the letter was stored in, the letters stored next to letter A, the home's mailbox, and mailboxes from homes on either side of the home.

Overall, the Trenton PDC processed approximately 5 million letters between October 9–October 16, and the Brentwood PDC processed 13 million letters between October 9 and October 21, when they were closed. Of these, approximately 1.1 million letters were processed at

each facility within 24 hours after the letters to Senators Daschle and Leahy passed through, half of which were bulk letters (Table 1).

Of 33 bulk mailing companies that sent letters through the Trenton PDC, 19 sent letters to Connecticut; 17 of these companies had no records of bulk letters sent to the address of the patient or her close contacts.

Postal Environmental Investigation

The Southern Connecticut PDC is approximately 350,000 square feet. It has 11 machines to cancel and code letters originating there and 18 machines to sort letters originating in and arriving at the Southern Connecticut PDC. Each letter-sorting machine has 48–52 columns of permanent collecting bins arranged in columns four bins high (Figure 2). Flats and parcels are processed by different machines located in different areas of the PDC. The facility is highly computerized and processes approximately 3 million letters, flats, and parcels daily.

Forty-one (7%) of 590 samples from the Southern Connecticut PDC yielded *B. anthracis.* The strain was indistinguishable from that of the bioterrorism-related isolates (9). All of the positive samples were from 4 (31%) of the 13 final sorting machines. Sorting machine 10, normally used to sort bulk letters as they arrived at the facility, was heavily contaminated. A vacuum sample from the vibrator-feeder area yielded anthrax spores, estimated as 2.9 million *B. anthracis* CFU in 0.53 g of paper dust collected. Wipe samples of 30 (58%) of 52 columns of bins from this machine yielded *B. anthracis*. Of 65 swab, wipe, or vacuum samples collected from the sorting machine (no. 6) that completed the final sort of letters that included the patient's mail carrier route, only the wipe sample taken from the column of bins that held sorted letters for the patient's postal carrier route were positive for *B. anthracis* (Table 2). Cultures of the 63 environmental samples taken at the local post office and 485 nasal swabs from employees working at the Southern Connecticut PDC or the local post office were negative for *B. anthracis*.

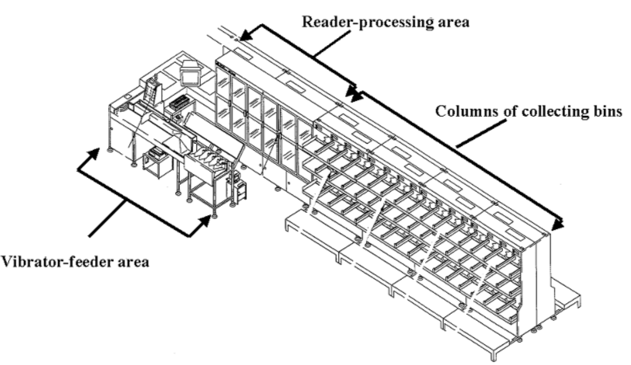

Figure 2. Diagram of a letter-sorting machine

Discussion

This report describes the epidemiologic and environmental investigation of a case of inhalational anthrax in a 94-year-old woman from rural Connecticut. Molecular subtyping and antibiotic susceptibility testing of the isolate from blood culture demonstrated that it was indistinguishable from the other bioterrorism-related isolates, establishing a link to the cases that were caused by the mailing of intentionally contaminated letters (3,7,8). Surveillance efforts indicate that this was an isolated case in Connecticut and not a sentinel for a larger exposure (9,10). Although a direct exposure was not found, our investigation indicates cross-contaminated bulk mail as the source of her exposure.

Of the 11 cases of bioterrorism-related inhalational anthrax, 9 with known exposures had incubation periods of 5 to 13 days (15,16). Because 36 days had passed since the last known intentionally contaminated letters (Daschle and Leahy letters) were postmarked and the onset of the Connecticut woman's symptoms, we considered many nonpostal potential sources of exposure: aerosolized release in her community; an intentional release at her home; and tampering of products, including pill bottles, inhalers, foods, spices, or misting devices. We also looked for similarities with the unexplained case in New York City. Extensive surveillance, epidemiologic investigation, and environmental sampling found no evidence to support these hypotheses.

We also wanted to determine whether the patient received an intentionally contaminated letter similar to the Daschle and Leahy letters, which mainly resulted in

Table 1. Volume of letters processed after the *Bacillus anthracis*–containing letters to Senators Thomas Daschle and Patrick Leahy in the Trenton, New Jersey,and Brentwood, District of Columbia, processing and distribution centers during two intervals, October 2001

	Trenton, NJ		Brentwood, DC	
Type of letter	Oct. 9–16	Oct. 10[a]	Oct. 9–21	Oct. 10[a]
Bulk letters	~3,000,000	~500,000	~6,000,000	~500,000
First-class letters	~2,000,000	~500,000	~7,000,000	~ 600,000
First-class letters to Southern Connecticut PDC[b]	20,451	3,645	24,181	3,836
First-class letters to patient's local post office	39	9	66	9

[a]Oct. 10 represents the volume of letters processed during the 24-hour period after the letters to Senators Daschle and Leahy were processed.
[b]PDC, postal processing and distribution center

Table 2. Number of positive and total samples by sampling location and date, regional processing and distribution center—Connecticut, 2001

Sampling location	Nov. 21 positive total (%)	Nov. 25 positive/ total (%)	Nov. 28 positive/ total (%)	Dec. 2 positive/total (%)
Letter-sorting machine 4	0/0	0/0	1/12 (8)	1/48 (2)
Letter-sorting machine 6	0/3 (0)	0/ 2 (0)	0/22 (0)	1/48 (2)
Letter-sorting machine 10	0/0	0/0	4/ 8 (50)	30/52 (58)
Letter-sorting machine 11	0/1 (0)	0/0	1/8 (13)	3/52 (6)
All letter-sorting machines (n=14)	0/6 (0)	0/4 (0)	0/99 (0)	0/0
All letter-canceling machines (n=11)	0/10 (0)	0/8 (0)	0/34 (0)	0/0
All flats-processing machines (n=4)	0/8 (0)	0/18 (0)	0/4 (0)	0/0
All parcel-processing machines (n=4)	0/10 (0)	0/2 (0)	0/4 (0)	0/0
Other locations in regional PDC	0/27 (0)	0/25 (0)	0/21 (0)	0/0

[a]Although multiple sampling techniques were used, exact locations were not sampled in a manner that would allow comparison of results by sampling techniques. Therefore, all types of samples are listed as a composite total. PDC, processing and distribution center.

inhalational anthrax cases, rather than an intentionally contaminated letter similar to the New York City media letters, which resulted solely in cutaneous cases (15). Environmental sampling of the Trenton and Brentwood PDCs and the Hart Senate Office Building, which processed or received the Daschle and Leahy letters, indicated widespread contamination in these locations (13,17). In contrast, environmental sampling showed only focal contamination in the Southern Connecticut PDC and no contamination in the patient's home, making receipt of a letter similar to the Daschle or Leahy letters unlikely. In addition, we identified no anthrax illness among postal employees at the Southern Connecticut PDC or the local post office (1,4). Although no cases of anthrax-related illness were identified in employees of the Hart Senate Office Building, early administration of postexposure chemoprophylaxis (17) likely prevented this.

The recovery of a cross-contaminated letter demonstrates the plausibility of cross-contaminated letters reaching their destination with demonstrable amounts of spores still on the envelope. Though *B. anthracis* was readily cultured from the outside of letter A's envelope, we found no evidence to indicate letter A was actively shedding or transferring spores at any point in Connecticut. This demonstrates that spores can remain on or embedded in the outside surface of an envelope without measurably contaminating the environment.

The 36-day gap from the Dashle and Leahy letters to the onset of the patient's symptoms suggests that her incubation period may have been longer than that seen with the first nine bioterrorism-related inhalational cases. In addition, the lack of any environmental evidence of *B. anthracis* spores in her home or any of the known locations she visited in the 60 days preceding illness suggests that her exposure dose was much lower than the 50% lethal dose cited in the literature (18). While we cannot exclude the possibility of an additional unrecognized intentional release, our findings are consistent with evidence from studies of inhalational anthrax in nonhuman primates (19).

We believe the patient received either a bulk letter that was directly cross-contaminated in the Trenton PDC or a bulk letter that was secondarily cross-contaminated in the Southern Connecticut PDC. Alternatively, we cannot exclude the possibility that the patient was exposed to a bulk letter secondarily cross-contaminated by one of the eight first-class letters sent to her postal route that were processed in the Trenton or Brentwood PDC after the Daschle and Leahy letters. The fact that she tore her bulk letters in half provides a possible mechanism for releasing a limited number of spores embedded in the surface of the envelope into her breathing space, a number that was too small to detect during environmental sampling of her home.

The possibility of cross-contaminated letters as a cause of anthrax illness has been postulated (20). Although cross-contaminated letters are potential sources of exposure and the risk of critical *B. anthracis* exposure through cross-contaminated letters is low, our investigation did not support earlier assumptions. Specifically, we found no evidence to indicate that a first generation cross-contaminated letter (letter A) was actively shedding or transferring spores at any point in Connecticut. Thus, cross-contaminated letters can have markedly different levels of cross-contamination and potential to shed spores. An individual assessment is necessary to determine the magnitude of risk.

If we assume that the case in Connecticut, and possibly the unexplained case of inhalational anthrax in New York City (21), resulted from exposure to cross-contaminated letters, the overall risk for inhalational anthrax from cross-contaminated letters appears very low. Approximately 2 million pieces of mail passed through the Trenton and Brentwood PDCs during the first 24 hours after the Daschle and Leahy letters contaminated these facilities, and approximately 18 million pieces passed through before the facilities were closed. This conclusion is consistent with the expectation that the majority of letters would not be heavily cross-contaminated and that only a limited

number of anthrax spores might remain on an envelope by the time it reaches its destination.

If low-dose exposure occurred through the mail, numerous persons might have had such exposures. Why illness developed in the Connecticut patient and not in others is unknown, but the reasons might include her habit of tearing mail in half before disposal, her advanced age, her history of obstructive lung disease, and her use of inhaled bronchodilators (3). If a future mailing of *B. anthracis* spores is recognized, persons could be advised to open letters in a well-ventilated area, avoid tearing envelopes, discard envelopes after opening, and wash hands after handling envelopes (9).

The finding of the approximate equivalent of 3 million spores from a vacuum sample on one sorting machine underscores the importance of maintaining the revised postal facility cleaning procedures geared to minimize aerosolization of dust (12). Before the mailing of intentionally contaminated letters, sorting machines were routinely cleaned by using compressed air. After investigators recognized that aerosolization of spores during cleaning might have contributed to inhalational anthrax cases among postal workers, vacuum cleaners with HEPA filters replaced compressed air cleaning of sorting machines (1,4,13).

A number of key limitations exist to this investigation, however. First, we do not know the threshold for detecting spores in the environment. The absence of positive cultures in the home and other places sampled in her town does not exclude the presence of *B. anthracis* spores in these locations. Nonetheless, locations that processed or received intentionally contaminated letters had widespread contamination (13,17). Also, because the patient's home was frequently vacuumed and dusted, measurable amounts of spores could have been removed during cleaning. We tried to assess this by testing contents of vacuum cleaner bags but were still unable to obtain positive cultures. Second, because we were unable to completely trace bulk letters, we do not know if she actually received any bulk letters that passed through the Trenton PDC. Third, the investigation began >1 month after anthrax spores were introduced into the postal system. Thus, certain potential environmental evidence might have disappeared by the time our investigation began.

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Isolated Case of Bioterrorism-related Inhalational Anthrax, New York City, 2001

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On October 31, 2001, in New York City, a 61-year-old female hospital employee who had acquired inhalational anthrax died after a 6-day illness. To determine sources of exposure and identify additional persons at risk, the New York City Department of Health, Centers for Disease Control and Prevention, and law enforcement authorities conducted an extensive investigation, which included interviewing contacts, examining personal effects, summarizing patient's use of mass transit, conducting active case finding and surveillance near her residence and at her workplace, and collecting samples from co-workers and the environment. We cultured all specimens for *Bacillus anthracis*. We found no additional cases of cutaneous or inhalational anthrax. The route of exposure remains unknown. All environmental samples were negative for *B. anthracis*. This first case of inhalational anthrax during the 2001 outbreak with no apparent direct link to contaminated mail emphasizes the need for close coordination between public health and law enforcement agencies during bioterrorism-related investigations.

After the World Trade Center attack on September 11, 2001, the possibility of bioterrorism in New York City (NYC) became a preeminent concern at the Department of Health (DOH). Active syndromic surveillance at emergency department for bioterrorism-related illnesses was initiated in 15 hospitals, and frequent broadcast alerts were sent by email and fax to all NYC emergency departments, commercial and hospital laboratories, infection-control programs, and selected providers (1).

After the announcement of the inhalational anthrax index case in Florida on October 4 and the cutaneous anthrax index case in NYC on October 12, DOH enhanced its active surveillance activities citywide (2). Detailed diagnostic and treatment protocols were provided through a broadcast alert system and the DOH website to the medical and laboratory community, including emergency departments, intensive-care units, infectious disease and infection-control specialists, dermatologists, and laboratories. A provider hotline was established for rapid referral and evaluation of suspect cases. Broadcast fax alerts also were sent to veterinarians to request reporting of suspect animal cases. In addition, the emergency department–based syndromic surveillance system was expanded to 29 hospitals to augment DOH's ability to detect a large, covert bioterrorist event. The medical examiner's office was asked to notify DOH of any suspicious deaths from unexplained sepsis or respiratory causes.

During October, four simultaneous investigations were conducted at news media outlets where cutaneous anthrax cases were detected among employees (M. Phillips, et al., unpub. data). All interviews were performed by teams of

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investigators from DOH, Centers for Disease Control and Prevention (CDC), and law enforcement on the basis of pre-established agreements between DOH and the New York field office of the Federal Bureau of Investigation (FBI), and its associated Joint Terrorism Task Force (a task force between the NYC Police Department and FBI). By the end of October 2001, seven laboratory-confirmed or suspected cutaneous anthrax cases had been reported in NYC. All case-patients were thought to have been exposed through direct contact with contaminated mail addressed to media outlets and postmarked on September 18 (3). The last known contaminated letters were postmarked on October 9 from Trenton, New Jersey, to Senators Thomas Daschle and Patrick Leahy in Washington, D.C.

Case Confirmation

On October 28, 2001, a local hospital reported a suspected case of inhalational anthrax to DOH. The case-patient was a 61-year-old female with a 3-day history of progressive weakness, chest heaviness, myalgia, cough, and shortness of breath. She was admitted to intensive care with respiratory failure, emergently intubated before being interviewed, and treated with multiple antibiotics and diuretics for a presumptive diagnosis of community-acquired pneumonia, congestive heart failure, or inhalational anthrax (4). On October 29, nonmotile, gram-positive rods in long chains were isolated from routine blood cultures, and her antibiotic therapy was adjusted to provide for enhanced coverage of inhalational anthrax. That evening, *Bacillus anthracis* was preliminarily identified from her blood culture isolate and from pleural, fluid, and bronchial washings by polymerase chain reaction (PCR) at the DOH Public Health Laboratory and CDC. The following day, pleural and blood isolates were confirmed as *B. anthracis* by gamma phage lysis and direct fluorescent antibody testing. The case-patient died on October 31. *B. anthracis* isolates were subtyped at CDC by multiple-locus variable-number tandem repeat analysis (MLVA) and sequencing of the *pag*A gene. All isolates were MLVA genotype 62 and *pag*A genotype I, the same genotype as all other isolates from the 2001 anthrax outbreak in Florida, New Jersey, Washington, D.C., and Connecticut (5).

We report the results of the epidemiologic and environmental investigation by DOH, CDC, and local and federal law enforcement agencies in response to this isolated case of inhalational anthrax. The objectives of our investigation were to determine the time, location, and route of exposure; to identify any additional cases of cutaneous or inhalational anthrax; to determine whether this case was an isolated case or sentinel case of a larger outbreak; and to guide our public health response.

Methods

Case Investigation

Immediately after confirming the case-patient's diagnosis, epidemiologists from DOH and CDC and a detective and special agents from the Joint Terrorism Task Force formed joint investigative teams to ensure the rapid and efficient sharing of relevant information between the epidemiologic and criminal investigations. To identify the time and location where the case-patient might have been exposed to anthrax during the 60 days before illness onset, detectives from the NYC Police Department and FBI along with local and federal epidemiologists performed joint interviews of the patient's social, work, and neighborhood contacts. We chose a 60-day period on the basis of the range of the inhalational anthrax incubation period during the Sverdlovsk anthrax outbreak in 1979 (6). We conducted regular interagency meetings to analyze new information collaboratively and strategize about the next steps of the investigations.

We collected information about the case-patient's habits and activities through interviews with co-workers, neighbors, acquaintances, and a mail carrier to uncover any potentially relevant personal details, including places she frequented, and her social contacts. Investigators searched the case-patient's apartment, examined personal effects, reviewed telephone and financial records, and visited four post offices that she was known or thought to have used. To locate persons who might have information regarding activities during the incubation period, we displayed the case-patient's photograph in churches that she reportedly attended and in Chinatown, which she frequented. Employees from 15 businesses near the case-patient's apartment complex and work were interviewed. Members of the NYC Anthrax Investigation Team also met with investigators of the other unexplained inhalational case in Connecticut (7).

Using the identification number from a subway transit card issued to the case-patient by the New York City Metropolitan Transportation Authority (MTA), we obtained data about her transit activity from October 22 to October 26, indicating which buses and subway stations she entered in the week before onset of illness. The MTA also identified a previous transit card number with boarding times that matched the pattern of subway use linked to her card. Using this information, we were able to approximate her bus and subway travel during the previous month (September 21–October 20).

Case Finding

Our investigation included active case finding and surveillance for additional anthrax cases at the case-patient's workplace and in her apartment complex. We asked all co-

workers, patients, and visitors who had spent >1 hour at her workplace during the preceding 2 weeks to report for an interview; at the interview, they were offered antibiotic prophylaxis. We also used the following information to identify additional suspect cases: 1) the hospital's employee health department list of all employees who had been evaluated for fever during the previous 2 weeks; 2) the human resources department list of employees who had missed >1 day of work during this period; 3) interviews regarding symptoms in occupants from 27 of 28 of the neighboring apartments in her complex; and 4) a community meeting in the case-patient's Bronx neighborhood in which the case was discussed and neighbors were encouraged to report illnesses or skin lesions suggestive of anthrax.

In addition, active surveillance for suspect cases was established with the U.S. Postal Service and MTA in NYC because of concern regarding potential exposures in post offices, from mail, or in the subway system. Any ill employee was contacted by DOH staff by telephone and asked about symptoms. Any suspect cases were referred for immediate evaluation and follow-up.

Environmental and Laboratory Investigation

To search for evidence of a recent exposure to aerosolized anthrax-containing particles, we collected both nasal swabs and environmental samples. On October 29, we collected nasal swabs from 28 co-workers who worked near the case-patient. We used four sampling techniques for environmental surface samples: dry Bacti-swab (Remel Inc., Lenexa, KS) sampling, wet swabs, composite dry swabs, and HEPA vacuum samples (8).

From October 29 through December 12, sampling was performed in and around the case-patient's apartment and from selected personal effects: from the hospital where she worked, with an emphasis on the mail bins, mailroom, and stockrooms she frequented; in an acquaintance's apartment where she had slept during the incubation period; from a neighborhood post office where she had purchased a postal money order 2 days before illness onset; from three mail-processing facilities and two post offices that served her home and workplace; and from two businesses near her apartment complex (Table). Subway stations were selected for sampling on the basis of the pattern of her subway use gleaned from her MTA transit card (Figure). Nasal swabs, bulk specimens, dry surface swabs, composite swabs, and vacuum sock samples were analyzed by the DOH Public Health Laboratory and the U.S. Department of Defense by using standard culture techniques and PCR analysis (9).

Mail Investigation

We obtained data from the U.S. Postal Service on the final destinations for first-class mail sorted from October 9 to October 16 on the same digital bar code–sorting machine in the Trenton, N.J., processing and distribution center that sorted the letters addressed to Senators Daschle and Leahy. Bulk mail is presorted and not labeled with codes. We focused on first-class mail that was then delivered to the same zip codes as our case-patient's home and work addresses.

Public Health and Prevention Activities

On October 30, in conjunction with the hospital, DOH established an antibiotic prophylaxis clinic for persons who were considered at potential risk for inhalational anthrax; at this time, we expanded the initial prophylaxis given to include the 28 staff persons who worked in the hospital basement near the stockroom and mailroom (10). All co-workers, patients, and visitors who had spent >1 hour at the case-patient's workplace in the preceding 2 weeks were recommended for antibiotic prophylaxis evaluation. Initially, persons were offered 10 days of doxycycline or ciprofloxacin (11), pending results from the epidemiologic and environmental investigations to determine whether the hospital was the site of exposure. We offered amoxicillin to children on the basis of the susceptibility pattern of the other *B. anthracis* isolates associated with this outbreak (following CDC guidelines) (12).

Results

Case Investigation

The case-patient was a resident of an apartment in a predominately Hispanic section of the Bronx; she lived alone. She had no known family members in NYC and a limited number of close acquaintances. A refugee from Vietnam, she had lived in the United States for 26 years. Her acquaintances at work and in her neighborhood reported her daily routine and usual activities to be habitual, with a regular work schedule, visits to Chinatown in Manhattan to shop, and trips to post offices and department stores near her workplace and home. Although reportedly friendly and generous, she lived a solitary life. Her apartment was clean and tidy. We collected no information suggesting that she had traveled outside NYC during the 60-day period before onset of illness. Neighbors did not recall any recent visitors to her home. From information received from MTA, we ascertained that she rode the No. 6 Lexington Avenue subway almost daily, traveling from her home in the Bronx to her workplace in Manhattan (Figure).

She had worked full time in an East Side Manhattan hospital for 12 years, delivering supplies from basement stockroom to clinics and wards within the hospital. She had not missed any work in the several weeks before onset of illness. She did not directly work with or sort mail. The hospital's mail was sorted in a section of one of these stockrooms, where it was placed into wooden mail slots.

Table. Summary of environmental test sampling in inhalational anthrax investigation, New York City, 2001[a]

Date in 2001	Location	Items	Wet swabs	Dry swabs	Vacuum filters
10/29	Case-patient's hospital workplace: old and new mail room	Mail cubby spaces, air intake, lights, desk top, computer keyboard, and air-conditioning air-intake filters		10	
	Case-patient's workplace: workroom, basement rooms, and elevator	Desk tops, floor, lighting and vents, door casings, walls, and ceilings		24	
	Admitting hospital	Case-patient's clothing and personal property from admission		7	
	Case-patient's workplace	Nasal swabs of hospital coworkers		28	
10/30	Case-patient's apartment	Appliance tops, mail bins, table tops, post office receipts, window sills, light fixtures, trash cans, and bank cards		40	
10/31	Case-patient's workplace locker and workspace	Locker contents (clothing, shoes, lab coat, personal belongings), elevator exhaust blades and cages, air-intake grills, door jambs, and computer cooling fans		52	4
11/1	Manhattan post office A	Computer fan intakes, light fixtures, teller computer monitors, air-intake grills, counter surfaces, and vacuum sample of multiple mail-sorting cubbies		12	1
	Case-patient's apartment	Mailbox, adjacent mailboxes, elevator fans, refrigerator, baseboards, personal items, clothes hampers, television screen, boxes containing recent mail, light fixtures, vacuum cleaner dust, and cooling fans		29	
	Case-patient's apartment	Closet, hanging garments, bedroom clothes, and hallway bureau			8
	Personal items from case-patient's apartment	Slippers, shoes, hats, pieces of recent mail, address book, pictures, and stuffed animal		9	
	Office of the Chief Medical Examiner	Autopsy room, sink, table, cutting floor, and floor		31	
11/2	Bronx post office A	Multiple mail slots, sorting areas, and intake grills of ventilation systems		15	3
	Manhattan post office B	Mail-sorting areas and ventilation system, fluorescent light fixtures		9	
11/3	Case-patient's workplace (basement storage)	Door jams, light fixtures, conduits, air-conditioner intake filters, pipes		8	
11/4	Bronx mail sorting and distribution center	Optical character readers, air intake of ventilation system, manual sorting stations, dead-letter repository, digital bar code sorters (including the machine that sorts mail to case-patient's home), overhead air filters		25	
	Bronx parcel post office B	Sorting stations, registered mail cage and delivery trucks		8	
	Case-patient's apartment	Powder samples		9	
11/5	Bronx post office box A	Case-patient's post office box		1	
	Friend's home	Dining room and bedroom surfaces		2	
	Case-patient's workplace (basement storage)	Storage lockers, urinal, plumbing, switches, and bulbs		7	
	Case-patient's workplace work station	Fan, light, and pipe		5	
11/6	Case-patient's personal effects from apartment	Unspecified substance in case-patient's wallet		1	
11/11	Subway line, #6 Lexington Ave line (including Grand Central Station) and 1 N/R station used by case-patient	Multiple dust-collecting areas along platform and multiple air-intake filters of recently installed air-conditioner system in Grand Central Station	120		19
	Four subway stations not known to be used by case-patient (control stations)	Multiple dust-collecting areas along platform	76		
11/30	Two businesses near to case-patient's apartment that received mail from Trenton postmarked October 9	Mail bins, scissors, letter openers, desk, phone, window, computer, air-conditioner, and copier		40	
12/12	Case-patient's personal effects from apartment associated with hypothesized exposure locations or pathways	Coat, shoes, tennis shoes, slippers, hats, scarf and lab coat, hospital identification card, documents dated in mid-October including mail, money orders, bank withdrawal slips, receipts, phone, and perfume bottles			18
Total			196	372	53

[a]Left column indicates the date samples were taken. Aggressive sampling was performed by intensively and individually vacuuming each item by using a HEPA filter vacuum sock. Collected material was processed through a membrane to selectively remove extraneous material (e.g. hairs, which can be saved for later forensic examination) and concentrate spores in the filtrate. Filtrate was then analyzed by polymerase chain reaction and cultured for *Bacilllus anthracis*. Material in perfume bottles was cultured directly. Tests on samples were completed on 12/12/2001.

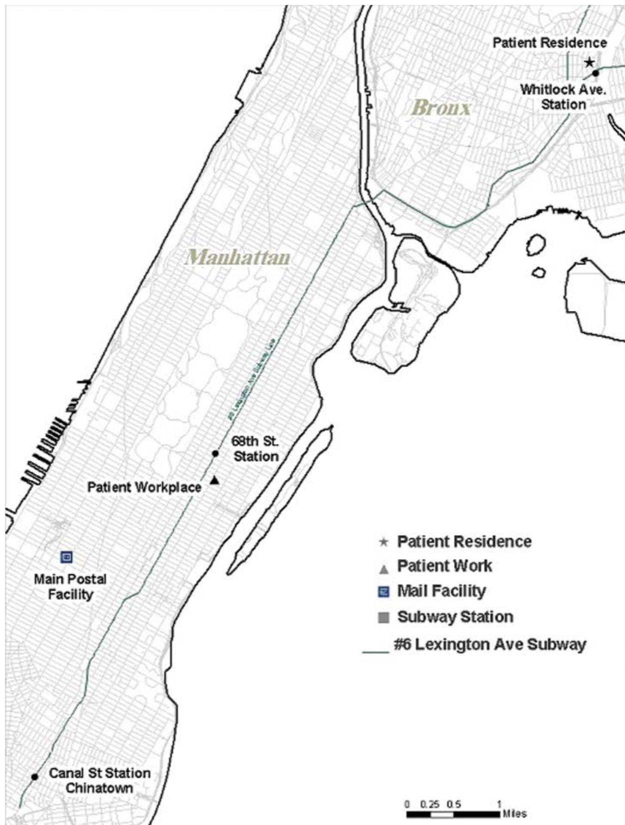

Figure. Key Manhattan and Bronx locations during investigation of inhalational anthrax, New York City, 2001.

The mailroom and stockroom staff reported no suspicious packages or letters.

We constructed a timeline of her activities in the several weeks preceding her illness. Although her subway transit card, work records, financial records, and sales receipts allowed us to account for some of her activities, approximately 40% of the nonwork-related hours before her death remained unaccounted for. The criminal investigation found no suspicious letters or activity to connect the case-patient to any of the known anthrax-laden postmarked letters, and no evidence existed that she had visited any of the media sites in NYC affected by this outbreak. Shared information with the investigators in Connecticut indicated that she and the case-patient in that state had little in common.

Case Finding

A total of 232 coworkers, occupants from 27 of 28 neighboring apartment units, 35 acquaintances, and 1,675 hospital patients and visitors were screened for symptoms of cutaneous or inhalational anthrax in connection with this single case. A total of 69 persons with respiratory symptoms and 21 with suspicious skin lesions were followed up by medical evaluation or telephone call. None of these persons were diagnosed with inhalational or cutaneous anthrax.

We contacted all hospital employees listed as ill on absentee (n=60) and employee health (n=88) lists and found them to have recovered from minor illnesses or injuries. Enhanced surveillance in NYC hospitals, the U.S. Postal Service, and emergency departments in NYC have continued since 2001 to 2003. No further cases of anthrax have been identified in NYC.

Environmental Investigation

All 621 environmental samples tested negative for *B. anthracis* by culture and PCR: workplace (n=138), apartment (n=86), personal effects (n=35), an acquaintance's apartment (n=2), businesses near her apartment (n=40), post offices (n=74), the Office of the Chief Medical Examiner (n=31), and subway (n=215) (Table).

Mail Investigation

The U.S. Postal Service identified letters delivered to the case-patient's work address, but not to her home address, that were postmarked on October 9 in Trenton and sorted by the same digital bar code–sorting machine as the letters sent to Senators Daschle and Leahy. However, all other hospitals with the same zip code as her workplace and many other places in the city also received mail postmarked on October 9 by this same machine. The closest location to her home where an October 9–postmarked letter from the Trenton facility was delivered was a commercial property two blocks from her apartment complex. No letters postmarked October 9 were identified at the two businesses located at this property, no persons at either business had been ill with symptoms suggestive of anthrax infection, and all environmental samples were negative.

Five digital bar code–sorting machines at the main Manhattan postal distribution center tested positive for *B. anthracis* in late October during the initial investigation of the media-related cutaneous cases. None of these five contaminated machines routinely performed final sorting of mail for the 5-digit zip codes that included the case-patient's home and workplace. The sorting machines that routinely sorted mail sent to the case-patient's workplace and home zip codes tested negative.

Public Health Intervention

On the recommendation of DOH, the local hospital where the case-patient worked closed voluntarily for 10 days after her diagnosis. DOH and local hospital screened and offered prophylaxis for anthrax to 232 coworkers and 1,675 hospital patients and visitors beginning on October 29. After all of the initial environmental samples at the case-patient's workplace tested negative and no additional suspect cases were identified, DOH recommended that

all persons discontinue antibiotic prophylaxis on November 7.

Discussion

This investigation focused on the first and only case of inhalational anthrax in NYC during the 2001 anthrax outbreak. This case was the first of two that occurred during this outbreak that did not have an apparent direct link to contaminated mail (13). No other confirmed cutaneous or inhalational anthrax infections associated with this case were identified during the 2001 outbreak. The timing, location, and route of the case-patient's exposure remain unknown. Our epidemiologic and environmental investigations yielded no firm indications regarding when, where, how, or why this case-patient was infected. Despite concern that she might represent the sentinel case of a much larger outbreak, subsequent surveillance showed that this case was probably an isolated case of inhalational anthrax and not part of a larger local outbreak.

During this investigation, we considered multiple hypothetical scenarios to explain how the case-patient could have been exposed to *B. anthracis*. Natural exposure was thought to be unlikely because the last case of naturally acquired anthrax in NYC occurred in 1947, the case-patient had no known risk factors for natural infection, and her isolate was genotypically indistinguishable from other isolates from the 2001 anthrax outbreak. Although exposure to an intentionally contaminated letter was considered the most likely hypothesis from the onset, our case-patient had none of the risk factors for anthrax infection identified among earlier cases (e.g., working for the news media or government, handling mail) (14). Other less likely explanations for her death—that she was associated with the terrorists or targeted purposely, that she was present when a small-scale attack intentionally occurred, or that she happened to pass by when a small amount of anthrax spores were accidentally released by the perpetrator—were considered, but no evidence supporting these hypotheses was discovered. In the absence of any data to support alternative hypotheses, and consistent with the hypothesis raised in the Connecticut case that contact with cross-contaminated bulk mail accounted for exposure (7), we think that this hypothesis is also reasonable for our NYC case. However we found no direct evidence to support or to refute it.

We cannot definitively conclude, however, that contaminated or cross-contaminated mail was the mechanism of exposure in this isolated case. First, the case-patient was not linked to a known contaminated or threat letter, and environmental testing did not provide any evidence of anthrax spores in her home or workplace. Second, although some potentially cross-contaminated mail from the contaminated digital bar code–sorting machine in Trenton was delivered to her workplace and to an address two blocks from her apartment, similar mail was sent to thousands of other locations in NYC, the metropolitan area, and nationwide. Only one other additional inhalational case was identified in the United States after this case (13). More cases of cutaneous or inhalational anthrax might be expected if cross-contaminated mail were the mechanism of transmission for our case-patient.

We also found no evidence of inhalational anthrax cases in the areas where the most heavily contaminated postal distributional centers were located in Washington, D.C., and New Jersey. Moreover, the two unexplained cases in NYC and Connecticut occurred several weeks after the last known contaminated letters were postmarked. Why cases that might have been caused by cross-contaminated mail did not occur closer to the time that cross-contamination likely occurred remains unclear. Although our surveillance may have missed a nonhospitalized person ill with anthrax, no other inhalational cases were identified nationwide. Therefore, if cross-contaminated mail was the source for these two last inhalational cases, the risk for illness after exposure to low levels of spores on secondarily contaminated mail is low, given that potentially millions of letters might have had low-level contamination (15) based on the positive environmental findings in numerous postal facilities during the 2001 anthrax outbreak.

We decided to close the case-patient's workplace several hours after her definitive diagnosis. Four factors were involved in this early decision: 1) whether the hospital was the site of recent aerosolized anthrax was unclear, 2) our case had no obvious link to exposure through the mail, 3) environmental testing was facilitated by a closed facility, and 4) closure put the fewest possible people at risk for further exposure. We benefited from decisions made in other states during other anthrax investigations in the preceding weeks. After the final results of the environmental sampling were available, the hospital was reopened on November 6.

Our entire investigation lasted 6 weeks and involved >100 local, state, and federal investigators. The bulk of the environmental sampling was completed within 7 days, although subway sampling began 2 weeks after the case-patient went to the hospital (Table). The case-patient's hospital workplace was not forced to close permanently, and employees were back at work 10 days after she was diagnosed.

Some limitations exist in our investigation of this case. The case-patient's rapid death prevented investigators from interviewing her directly to assess potential sources of exposure, including contact with suspicious mail or persons. Given the absence of additional inhalational cases in NYC for comparison, we could only speculate about the activities that represented risk factors for her infection. Unlike the subsequent Connecticut inhalational anthrax

case, we could not account for a large proportion of our case-patient's time when exposure could have occurred. Low-level contamination from mail in her home, workplace, or NYC postal facilities may also have been cleaned up before environmental testing during the 16-day period after these letters were processed. Finally, our environmental sampling strategy was based on the assumption that an aerosol release of *B. anthracis* at any of the locations would have resulted in evidence of environmental contamination detectable by the methods used.

All aspects of the epidemiologic and environmental investigation (e.g., interviewing, collecting samples, interpreting results, developing strategy) occurred through active collaboration with multiple local and federal partners, including public health and law enforcement agencies and the involved healthcare facilities. Our investigation underscores how pre-existing relationships and preplanning among local response agencies fosters the coordination and collaboration needed between the parallel epidemiologic and criminal investigations during an acute crisis (16).

This case also emphasizes the important roles that epidemiologic and environmental investigations play in shaping decisions regarding appropriate antibiotic prophylaxis during suspected bioterrorist events. Co-workers, patients, and hospital visitors were initially offered antibiotic prophylaxis when we were uncertain whether an aerosolized release had occurred in her workplace. Given the short incubation period for inhalational disease and the high death rate if treatment is not started early, we decided to initially offer antibiotic prophylaxis to potentially exposed persons. After the initial investigation failed to identify additional suspected cases or any evidence of anthrax spores in the case-patient's work environment, we recommended discontinuing antibiotics. If any of the environmental samples collected from her workplace had yielded *B. anthracis* isolates, we would have extended the duration of prophylaxis to $\geq$60 days to those persons deemed to be at risk for inhalational disease on the basis of the location of the positive results. The liberal use of broad antibiotic prophylaxis earlier in the 2001 anthrax outbreak is thought to have prevented many cases of inhalational anthrax (17).

Our investigation provided a number of lessons for future such investigations. We learned that a rapid and adaptive decision-making process regarding scientific issues is necessary and that this process must use both governmental and academic advisors. Laboratory surge capacity is required to continue vital public health activities and respond to a crisis, and surveillance systems for bioterrorism incidents are needed that can be used routinely with familiarity by staff. Health department staff must be familiar with environmental testing and decontamination; proactive information management with early media presence and key officials who are informed is essential.

DOH, in cooperation with multiple local and federal agencies, investigated a single, fatal case of inhalational anthrax whose genotypic similarity to other cases of intentional anthrax in the 2001 anthrax outbreak indicates it was bioterrorism-related. The place and route of exposure to *B. anthracis* in this isolated case remain unknown. Unless the criminal investigation yields further answers, we will likely never know the source of infection or method of exposure in this case. This case highlights the challenges in explaining single bioterrorism-related illnesses with standard epidemiologic and environmental methods and underscores the need for coordination between public health officials and law enforcement agencies during bioterrorism-related investigations.

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Relapsing Fever–Like Spirochetes Infecting European Vector Tick of Lyme Disease Agent

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To determine whether relapsing fever–like spirochetes associated with hard ticks may infect *Ixodes ricinus* ticks in central Europe, we screened questing ticks for 16S rDNA similar to that of Asian and American relapsing fever–like spirochetes. We compared the prevalence of these spirochetes to that of Lyme disease spirochetes transmitted by the same vector. Relapsing fever-like spirochetes infect 3.5% of questing vector ticks in our three central European sites near the Rhein Valley. These spirochetes differ genetically from their American and Asian analogs while being relatively homogeneous in the region we sampled. The Lyme disease genospecies most commonly detected in central Europe are distributed broadly, whereas those that are less frequently found appear to be place-specific. The absence of co-infected ticks suggests that relapsing fever–like and Lyme disease spirochetes may not share hosts. Exposure risk for relapsing fever–like spirochetes is similar to that of certain Lyme disease genospecies. Although many persons may be bitten by ticks infected by relapsing fever–like spirochetes, health implications remain unknown.

Diverse pathogens infect ticks of the *Ixodes ricinus* complex in North America, Asia, and Europe. Viral agents include those of tick-borne encephalitis, Russian spring-summer encephalitis, louping ill, Powassan encephalitis (1), and its variant known as deer tick virus (2). The piroplasms that infect these ticks include *Babesia divergens*, *B. microti*, *B. odocoilei*, and *B. gibsoni* (3). Other infectious agents include *Ehrlichia (Anaplasma) phagocytophila*, *Rickettsia helvetica*, possibly *Bartonella quintana*, and a nonpathogenic endosymbiontic rickettsia (4–6). Entomophagic fungal, nematode, and wasp pathogens are also found in these ticks (7). The diverse array of Lyme disease spirochetes infecting ticks in the *I. ricinus* complex has been subdivided into at least six known genospecies, *Borrelia burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. bissettii*, and *B. lusitaniae* (8,9). More recently, a relapsing fever–like spirochete, *B. miyamotoi* (10), has been added to this array of parasitic microbes.

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B. miyamotoi was originally described from *I. persulcatus* ticks sampled in Japan. Its flagellin and 16S rRNA sequences resemble those of the relapsing fever spirochetes more closely than those of any of the known Lyme disease spirochetes (10,11). A closely related agent, designated as *Borrelia* nov. sp., has recently been discovered in North America (12). The primers used to detect infection by Lyme disease spirochetes generally fail to amplify DNA from these organisms. Although *Borrelia* nov. sp. infects approximately 2% of the American *I. scapularis* ticks, Lyme disease spirochetes are found in approximately 12%. The prevalence of *B. miyamotoi* infection in Japan, however, has not been determined. Whether these potential threats to human health are present in Europe remains unknown.

Relapsing fever–like spirochetes associated with hard ticks may infect European *I. ricinus* ticks. To determine whether such organisms are present in Europe, we screened questing ticks for DNA similar to that of the American and Asian relapsing fever–like spirochetes. In particular, we amplified a fragment of the 16S rRNA gene found in the various genospecies of Lyme disease spirochetes as well as in the Asian and American relapsing fever–like spirochetes and estimated how prevalent such infections are in a region in central Europe.

Materials and Methods

We sampled questing *I. ricinus* ticks from three sites located near the Rhein Valley in Germany and France. The German site was near the town of Maikammer and the French site was near the town of Lembach, approximately 40 km southwest of Maikammer. A second French site, Petite Camargue Alsacienne, was situated 160 km farther south, near the German and Swiss borders (13). Ticks were collected in April 2001 by passing a flannel flag over vegetation. Ticks were confined in screened vials and stored at 10°C ± 1°C, and all nymphs and adults were identified to species.

To detect and identify the various Lyme disease spirochetes and relapsing fever–like spirochetes in these ticks, the opisthosoma of each was opened in a drop of physiologic saline, and the midgut was obtained and transferred to a tube containing 180 µL lysis buffer (ATL Tissue Lysis Buffer,

Qiagen GmbH, Hilden, Germany) and 20 µL proteinase K (600 mAU/mg). Midguts were lysed at 56°C overnight. DNA was extracted by using the QIAamp DNA Mini Kit (Qiagen GmbH) according to the manufacturer's instructions. DNA of nymphal or adult ticks was eluted with 50 µL or 75 µL elution buffer, respectively, and stored at –20°C until polymerase chain reaction (PCR) was performed.

Borrelia genospecies were characterized by amplifying and sequencing a 600-nucleotide fragment of the gene encoding the 16S rRNA. To increase the sensitivity for spirochetal DNA detection in ticks, we used nested PCR. Aliquots of DNA suspensions (2 µL) were diluted to 50 µL by using 200 µM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 0.5 U Taq polymerase (Qiagen GmbH) as well as 15 pmol of the outer primer pair and PCR-buffer supplied with the Taq polymerase. We used the following primer sequences of the 16S rRNA gene as outer primers (14): 16S1A 5′-CTA ACG CTG GCA GTG CGT CTT AAG C-3′ and 16S1B 5′-AGC GTC AGT CTT GAC CCA GAA GTT C-3′ (positions 36–757). The mixture was placed in a thermocycler (PTC 200, MJResearch, Biozym Diagnostic, Hess. Oldendorf, Germany), heated for 1 min at 94°C, and subjected to 30 cycles of 20 s denaturation at 94°C, 20 s each for the first annealing reaction at 63°C with a 40 s extension at 72°C and a final extension for 2 min at 72°C. After the first amplification with the outer set of primers, 2 µL of the amplification product was transferred to a fresh tube containing 48 µL of the reaction mixture previously described, except that 2.5 mM $MgCl_2$ and 20 pmol of the inner primer pair was used 16S2A 5′-AGT CAA ACG GGA TGT AGC AAT AC-3′ and 16S2B 5′-GGT ATT CTT TCT GAT ATC AAC AG-3′ (positions 66–720). This mixture was subjected to 35 amplification cycles by using the cycle conditions described previously, except that the annealing reaction was performed at 56°C and the extension reaction lasted 30 s. DNA was extracted, reaction vials were prepared for amplification, templates were added, and products underwent electrophoresis in separate rooms. In each sixth reaction mix, water was added instead of extracted DNA to serve as negative control. PCR products were detected by electrophoresis in a 1.5% agarose gel stained with ethidium bromide.

Each PCR amplification product was purified by using a QIAquick-Spin PCR column (Qiagen GmbH) according to the manufacturer's instructions. Amplified DNA fragments were directly sequenced in both directions by using the inner primers by the dideoxynucleotide chain-termination method on a Li-COR DNA4200 sequencer (Li-COR Biosciences, Bad Homburg, Germany). Each resulting sequence was compared with sequences of the same gene fragment representing various spirochetal genospecies. The following sequences served for comparison: accession nos. X85196 and X85203 for *B. burgdorferi* s.s.; X85190, X85192, and X85194 for *B. afzelii*; X85193, X85199, and M64311 for *B. garinii*; X98228 and X98229 for *B. lusitaniae*; AJ224138 for *B. bissettii*; X98232 and X98233 for *B. valaisiana*; AY024345 for the American *Borrelia* sp.nov.; and D45192 for *B. miyamotoi*. A complete match, permitting no more than two nucleotide changes, was required.

For comparison with the American and Asian relapsing fever–like spirochetes, *Borrelia* genus–specific primers (Bf1 5′-GCT GGC AGT GCG TCT TAA GC-3′ and Br1 5′-GCT TCG GGT ACT CTC AAC TC-3′) were used to amplify and sequence a fragment of the 16S rRNA gene approximately 1,350-bp in length, according to the published PCR conditions (15). A set of degenerate primers (FLA120F 5′-AGA ATT AAT MGH GCW TCT GAT GAT G-3′ and FLA920R 5′-TGC YAC AAY HTC ATC TGT CAT T-3′) was used to amplify and sequence an approximately 800-bp fragment of the flagellin gene, according to the published PCR conditions (12).

Results

To determine whether relapsing fever–like spirochetes may infect *I. ricinus* ticks in our study sites, DNA of field-derived nymphs and adults was amplified by using primers that bind to a 16S rRNA gene fragment present in relapsing fever–like spirochetes as well as Lyme disease spirochetes. Of the 565 ticks that were processed, 197 produced amplification products. Of these, the sequences of 177 were consistent with those of known genospecies of the Lyme disease spirochete. Derived sequences from the remaining 20 samples corresponded closely to sequences of the Asian and American relapsing fever–like spirochetes. These DNA fragments (GenBank accession no. AY253149) differed by six bases from the American and Asian relapsing fever–like spirochetes. In all three sites, DNA of bacteria related to relapsing fever spirochetes was detected in questing *I. ricinus* ticks.

We estimated the prevalence of the various spirochetal infections in ticks sampled in our study sites. Spirochetes infected approximately a quarter of nymphal and more than a third of adult ticks (Table 1); adults were infected more frequently than were nymphs ($p=0.0001$, Fisher exact test). Approximately 3.5% of these questing *I. ricinus* ticks were infected by a relapsing fever–like spirochete, constituting approximately one tenth of all spirochete-infected ticks (10.2%). The prevalence of infection by the relapsing fever–like spirochetes was similar at each of the three sites. Although the relapsing fever–like spirochetes appeared to infect more adult than nymphal ticks, this difference was not significant. A relapsing fever–like spirochete commonly infects questing *I. ricinus* ticks in central Europe.

The relatedness of the European relapsing fever–like spirochete to such spirochetes from Asia and North

Table 1. Prevalence of spirochetal variants in questing *Ixodes ricinus* ticks sampled from central Europe[a]

Site	Ticks examined			% infected ticks infected by *Borrelia*						% total ticks infected by RFS
	Stage	No.	% infected	RFS	afz	gar	val	bur	lus	
PC	Nymph	107	26.2	10.7	28.6	28.6	32.1	7.1	0	2.8
	Adult	34	52.9	16.7	33.3	22.2	27.8	5.6	0	8.8
MK	Nymph	63	28.6	5.6	27.8	55.6	5.6	11.1	0	1.6
	Adult	113	38.9	13.6	22.7	31.8	29.5	2.3	0	5.3
LB	Nymph	45	17.7	0	62.5	12.5	0	0	25.0	0
	Adult	203	39.9	8.6	37.0	25.9	7.4	1.2	22.2	3.4

[a]The sampling sites were Petite Camargue Alsacienne, France (PC), Maikammer, Germany (MK), and Lembach, France (LB), and the variants were *B. afzelii* (afz), *B. garinii* (gar), *B. valaisiana* (val), *B. burgdorferi* s.s. (bur), and *B. lusitaniae* (lus), European relapsing fever–like spirochete (RFS).

America was determined by comparing a 780-bp fragment of the flagellin gene as well as a 1,106-bp fragment of the 16S rRNA gene. The flagellin sequence of the European relapsing fever–like spirochete (GenBank accession no. AF529084) was 98.8% similar to that of the American spirochete and 98.3% similar to that from Japan (Table 2). The European spirochetes were characterized by six unique nucleotides. The 16S rDNA sequence of the European variant (GenBank accession no. AF529085), similarly, shared 99.6% and 99.5% of the sequence of the North American and Japanese spirochete, respectively (Table 3). The flagellin and 16S rDNA sequences of the various European relapsing fever–like spirochetes were identical. The European relapsing fever–like spirochete differs from its American and Asian relatives and is relatively homogeneous within the region.

We described the relative prevalence of each genospecies in our sample of ticks. *B. afzelii* and *B. garinii* were most prevalent, each infecting approximately a third of all spirochete-infected ticks, regardless of location (Table 1). *B. burgdorferi* s.s., in contrast, infected few such ticks. The prevalence of *B. valaisiana* varied among our study sites; this spirochete appeared to be more prevalent in the sites that were considered southern ($p<0.05$, Fisher exact test). The range of *B. lusitaniae* was more restricted than that of the other genospecies. The genospecies of Lyme disease spirochetes that are most commonly detected in central Europe are distributed broadly, whereas those that are found less frequently tend to be place-specific.

Finally, we determined how frequently ticks might be infected by both relapsing fever–like and Lyme disease spirochetes. Of the 565 questing ticks examined, 7 (1.2%) harbored more than one spirochete variant. Mixed infections were distributed similarly among our study sites. *B. garinii* and *B. valaisiana* appeared to be associated with each other, co-infecting three adult ticks, as were *B. afzelii* and *B. burgdorferi* s.s., co-infecting two nymphs and one adult. Another adult tick harbored *B. garinii* and *B. afzelii*. No co-infections of relapsing fever–like and Lyme disease spirochetes were noted. The absence of an association between relapsing fever–like spirochetes and Lyme disease spirochetes in ticks suggests that these spirochetes may not share common hosts.

Discussion

Among the many microbes transmitted by the vectors of Lyme disease spirochetes are distinct *Borrelia* related to relapsing–fever spirochetes. *B. miyamotoi* infects *I. persulcatus* ticks in Japan, and a closely related spirochete infects a related tick on the East Coast of North America (10,12). We now know that a third member of this group infects *I. ricinus* ticks in central Europe. Despite their wide distribution, the three relapsing fever–like spirochetes appear to vary little; few nucleotides are substituted in the sequences of the two gene fragments that were examined. The relapsing fever–like spirochetes appear to be homogeneous in North America (12) as well as in Europe. We conclude that each of the various kinds of ticks that serve as vectors for Lyme disease spirochetes, *I. ricinus*, *I. persulcatus*, *I. scapularis* (= *dammini*), may be infected by relapsing fever–like spirochetes.

The recently identified relapsing fever–like spirochetes appear to co-exist regionally with the agents of Lyme disease and may inflate estimates of exposure risk to Lyme disease spirochetes on the basis of their prevalence in vector ticks. Neither darkfield nor immunofluorescence microscopy serves to discriminate Lyme disease spirochetes from relapsing fever–like spirochetes (12). By amplifying and sequencing a particular fragment of the 16S rRNA gene, Lyme disease spirochetes may be distinguished from relapsing fever–like spirochetes and identified to the genospecies level. Relapsing fever–like spirochetes constitute approximately one tenth of spirochetes infecting North American vector ticks (12) as well as those in central Europe. Questing *I. ricinus* ticks in our study sites more frequently harbor relapsing fever–like spirochetes than *B. burgdorferi* s.s. ($p<0.05$, Fisher exact test). The proportion of relapsing fever–like spirochetes appears to be similar in the population of European and American vector ticks.

The vertebrate host associations of relapsing fever–like spirochetes transmitted by hard ticks have not yet been defined. If the prevalence of a tick-borne pathogen increases with each developmental stage of the tick, a reservoir host is likely involved in its transmission cycle. Although evidence of such a pattern of accumulation, in the case of the relapsing fever–like spirochetes, is no more than marginal; field, as well as laboratory, observations suggest the

Table 2. Similarity matrix for the flagellin sequences of various relapsing fever and relapsing fever–like spirochetes (RFS)

Species	Variant	Accession no.	% similarity with					
			American RFS	European RFS	*Borrelia miyamotoi*	*B. lonestari*	*B. hispanica*	"Spain" strain
American RFS	MP2000	AY024344	100					
European RFS	LB-M56	AF529084	98.84	100				
B. miyamotoi	HT31	D43777	98.56	98.32	100			
B. lonestari	Texas	U26704	89.66	89.17	89.98	100		
B. hispanica	N.n.[a]	U28498	85.60	84.90	85.82	84.36	100	
"Spain" strain	N.n.	U28499	88.35	88.06	88.94	86.86	94.32	100

[a]N.n., not named.

Table 3. Similarity matrix for the 16S rRNA sequences of various relapsing fever and relapsing fever–like spirochetes (RFS)

Species	Variant	Accession no.	% similarity with					
			American RFS	European RFS	*Borrelia miyamotoi*	*B. lonestari*	*B. hispanica*	"Spain" strain
American RFS	MP2000	AY024345	100					
European RFS	LB-W100	AF529085	99.64	100				
B. miyamotoi	HT31	D45192	99.34	99.46	100			
B. lonestari	Texas-20	U23211	98.19	98.28	98.19	100		
B. hispanica	UESV/246	U42294	98.16	98.37	97.79	98.13	100	
"Spain" strain	N.n.[a]	U28502	97.10	97.56	96.69	97.06	98.87	100

[a]N.n., not named.

possibility of vertebrate hosts. *B. miyamotoi* has been isolated from the blood of a Small Japanese Field Mouse (*Apodemus argenteus*) (10). Infectivity of such naturally infected animals to ticks, however, has not been demonstrated. White-footed Mice (*Peromyscus leucopus*) experimentally infected by tick bites occasionally infect other ticks (12). Relapsing fever–like spirochetes are transmitted less frequently than Lyme disease spirochetes that cycle between the same reservoir and vector. Perpetuation of the relapsing fever–like spirochetes may not require a vertebrate reservoir.

To the extent that diverse pathogens share reservoir hosts, they might also co-infect their vector hosts. Although various Lyme disease spirochetes occasionally are detected in the same tick, the relapsing fever–like spirochetes appear not to co-infect ticks that are infected by Lyme disease spirochetes. None of our 565 European ticks and only 1 of 712 American ticks in an earlier study were co-infected in this manner (12). If these infections were distributed randomly, numerous co-infections would be anticipated. The presence of one kind of spirochete, however, appears to exclude the presence of the other. Lyme disease and relapsing fever–like spirochetes may directly interact in their tick or vertebrate hosts (e.g., inhibiting the other's proliferation) or each may be closely associated with a reservoir host that is zooprophylactic for the other kind of spirochete. Co-infection between diverse spirochetes occurs only infrequently.

The distribution of *B. lusitaniae*, the most recently described Lyme disease genospecies, is anomalous. With the possible exception of *B. valaisiana*, the prevalence of the other genospecies is constant from site to site. Indeed, these estimates correspond closely to those in a survey conducted in more northerly sites (16,17). *B. lusitaniae*, in contrast, was detected in only one of the three sites surveyed in the present study and not at all in four other sites that we have investigated (unpub. data). Although this genospecies may more frequently infect ticks in the Mediterranean basin (Portugal [18], Spain [19], and Tunisia [20]), it has been detected in ticks as far east as Slovakia (21), Moldovia, the Czech Republic, and Ukraine (22). The prevalence of *B. lusitaniae* in our Lembach site corresponds more to that in southern than in eastern Europe. Although *B. lusitaniae* induces disease when injected into susceptible mice (23), nothing is known about its pathogenic potential in people. The distribution of *B. lusitaniae* is irregular and its biologic relationships largely unknown.

Certain members of the relapsing fever group of spirochetes, mainly those transmitted by soft ticks, cause severe human disease. The classic agent of human relapsing fever in Europe is attributed to infection by *B. hispanica,* a relatively rare *Ornithodoros*-transmitted spirochete endemic to the southern part of that continent (4). Recently, a "Spain strain" of such spirochetes was implicated in human disease (24). The relapsing fever–like spirochetes that we describe differ fundamentally from these pathogens in that they are transmitted by hard ticks. Two kinds of spirochetes in this group are endemic to North America, *B. theileri* and *B. lonestari*, which are transmitted mainly by *Boophilus* and *Amblyomma* ticks, respectively. *B. theileri* is the agent of bovine borreliosis, a severe veterinary disease (25). *B. lonestari* appears to cause a human illness, known as Master's disease or southern tick-associated rash illness (STARI) (26–29). Although the Asian, American,

and European *Ixodes*-borne relapsing fever–like spirochetes relate more closely to each other than to *B. theileri* and *B. lonestari*, they form a monophyletic clade with these other hard-tick–associated spirochetes (12,30). Neither the pathognomonic recurrent fever of the soft-tick–transmitted spirochetes nor the transient fever and rash associated with Master's disease has been associated with infection by the *Ixodes*-borne relapsing fever–like spirochete. Exposure risk of this agent is almost as great as that to one or another of the European Lyme disease genospecies. Although many people may be bitten by ticks infected by relapsing fever–like spirochetes, the resulting health implications remain unknown.

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Leptospirosis in "Eco-Challenge" Athletes, Malaysian Borneo, 2000

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Adventure travel is becoming more popular, increasing the likelihood of contact with unusual pathogens. We investigated an outbreak of leptospirosis in "Eco-Challenge" multisport race athletes to determine illness etiology and implement public health measures. Of 304 athletes, we contacted 189 (62%) from the United States and 26 other countries. Eighty (42%) athletes met our case definition. Twenty-nine (36%) case-patients were hospitalized; none died. Logistic regression showed swimming in the Segama River (relative risk [RR]=2.0; 95% confidence interval [CI]=1.3 to 3.1) to be an independent risk factor. Twenty-six (68%) of 38 case-patients tested positive for leptospiral antibodies. Taking doxycycline before or during the race was protective (RR=0.4, 95% CI=0.2 to 1.2) for the 20 athletes who reported using it. Increased adventure travel may lead to more frequent exposure to leptospires, and preexposure chemoprophylaxis for leptospirosis (200 mg oral doxycycline/week) may decrease illness risk. Efforts are needed to inform adventure travel participants of unique infections such as leptospirosis.

Each year, 60 million Americans travel abroad. Increasingly, these persons are traveling to more remote and exotic destinations. Adventure travel is now the largest growing segment of the leisure travel industry, with a growth rate of 10% per year since 1985 (Adventure Travel Society, pers. comm.). This travel has led to an increasing risk for contact with pathogens uncommon in industrialized countries, especially for participants in adventure sports and extreme travel. Both of these pursuits may predispose persons to infection with unusual organisms through exposures to lakes, rivers, caves, and canyons, as well as insect vectors. These illnesses may be unfamiliar to practitioners in the travelers' home countries, and symptoms may go unrecognized. Leptospirosis, a bacterial zoonotic infection, is more frequently found in developing countries, and its protean early symptoms may be difficult to diagnose clinically. Prevention of leptospirosis in humans has previously relied on mechanical barriers and avoidance (1), but limited published data suggest that preexposure chemoprophylaxis may be beneficial to some groups (2–4).

From September 7 to September 11, 2000, the Idaho Department of Health, the Los Angeles County Department of Health Services, and the GeoSentinel Network (an international surveillance network of travel clinics) notified the Centers for Disease Control and Prevention (CDC) of at least 20 cases of febrile illness. The illness was characterized by the acute onset of high fever, chills, headache, and myalgias; major laboratory test abnormalities and important pulmonary or central nervous system involvement were absent. All ill persons had participated in the Eco-Challenge-Sabah 2000 multisport endurance race, held in Malaysian Borneo August 21–September 1, 2000. Three hundred four athletes from 26 countries and 29 U.S. states competed in the 10-day endurance event. Segments of the event included jungle trekking, prolonged swimming and kayaking (both in fresh and ocean water), spelunking (caving), climbing, and mountain biking. Symptoms and exposure history, as well as initial laboratory testing, suggested that the illness was leptospirosis. We undertook an investigation to determine the etiology of the illness in the athletes and to make public health recommendations. We report on the results of this investigation and discuss recommendations for preventing leptospirosis in adventure travelers.

Methods

Epidemiologic Investigation

Upon identification of the first ill athletes, a complete list of U.S. and international participants in the Eco-

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Challenge event, including their telephone numbers and email addresses, was obtained from the race organizers. In addition, race organizers and some athletes were interviewed to determine details of the race activities, geography of the course, and possible exposures. Athletes in the United States were contacted by telephone, either by CDC or by representatives of the athletes' state or local health departments, between September 7 and October 30, 2000. International athletes were contacted by representatives from their local ministries of health after notification by local GeoSentinel sites or the World Health Organization.

A standardized telephone questionnaire was administered, directed at determining demographics, symptoms, duration of illness, previous antibiotic use, and various exposures encountered during the event. A clinical case of illness was defined as onset of self-reported fever between August 21 and September 30, 2000, in an Eco-Challenge athlete, along with two or more of the following symptoms: chills, myalgias, headache, diarrhea, or conjunctivitis. We compared clinical case-patients with controls from the cohort of athletes to identify risk factors for illness.

Laboratory Investigations

Thirty-eight serum samples were obtained from a convenience sample of the cohort (who met the clinical case definition) for laboratory testing for various pathogens. All submitted samples were tested for *Leptospira*-specific immunoglobulin (Ig) M antibodies by dot-enzyme-linked immunosorbent assay (ELISA) dipstick (Dip-S-Ticks immunoassay, Integrated Diagnostics, Baltimore, MD) or by microplate IgM-ELISA (PanBio Ltd., Brisbane, Australia), according to the manufacturer's instructions. Laboratory evidence for leptospirosis was defined as a positive result for *Leptospira*-specific IgM antibodies.

All 38 samples were subsequently tested by microscopic agglutination test (MAT) by using a standard method (5), with 23 live antigen suspensions representing 17 serogroups. A titer of $\geq$200 against any of the antigens was considered positive evidence for a probable case of leptospirosis.

Given the broad differential diagnosis for febrile illness in the tropics, 18 submitted samples were also tested for alternative organisms. Samples with antibody responses to *Orientia tsutsugamushi*, *Rickettsia prowazekii,* and *R. typhi* were assayed by using a variation of the standard microimmunofluorescence test for rickettsiae (6). Testing for flaviviruses (dengue, Japanese encephalitis, Powassan/tick-borne encephalitis), alphaviruses (Chikungunya), bunyaviruses (Snowshoe Hare virus), and hantaviruses (Sin Nombre) was performed with the standard IgM ELISA assay (7).

Statistical Analysis

Results of the telephone survey were entered into EpiInfo v. 6.04b (CDC, Atlanta, GA); the dataset was then imported into SAS v. 6.12 and v. 8.2 (SAS Institute Inc., Cary, NC) for subsequent analysis. Because laboratory testing was only available from a subset of the cohort, athletes were categorized on the basis of the clinical case definition. All variables were examined using PROC GENMOD (SAS). Those factors significant by univariate analysis, as well as known risk factors, were examined in a multivariable logistic regression model. Colinearity and interaction among variables were calculated by using standard statistical techniques.

Results

Epidemiologic Investigation

Of the 304 athletes competing in the Eco-Challenge event, 189 (62%) were contacted, including 129 (92%) of the 140 U.S.-based athletes. The median age of the contacted cohort was 34 years (range 21–50 years); 94% were white, and 74% were men. No significant differences were found between ill athletes who met the case definition and non-ill athletes by age, race, or gender. The most common symptoms athletes reported included fever, chills, muscle aches, headache, and diarrhea (Table 1). Conjunctival suffusion, a hallmark finding in persons infected with leptospires, was reported by 40 (21%) athletes. Joint aches and calf/leg pain were also frequently reported. The status of athletes with respect to residence in an area endemic for leptospirosis was unknown.

Of the 189 athletes contacted, 80 (42%) met the clinical case definition. The median interval between the start of the race and onset of fever was 15 days (range 1–24 days), with a peak onset of fever on September 4, 2000 (Figure); no cases were detected after September 13. The median duration of illness was 7 days (range 1–17 days); 29 (36%) of the athletes who met the clinical case definition were hospitalized. No deaths were reported. Jaundice, pulmonary hemorrhage, meningoencephalitis, or other severe manifestations of leptospirosis were not reported.

Table 1. Self-reported clinical symptoms in Eco-Challenge athletes

Symptom	% (n=189)
Chills	50
Muscle aches	50
Fever	49
Headache	47
Diarrhea	33
Joint aches	30
Calf/leg pain	30
Red eyes	22

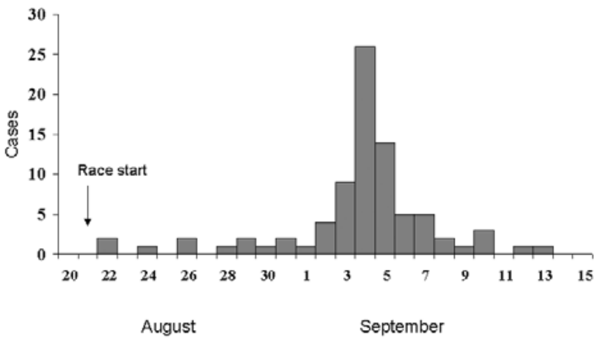

Figure. Date of fever onset for suspected and confirmed cases of leptospirosis in Eco-Challenge-Sabah 2000 athletes, Malaysian Borneo, August 21–September 14, 2000.

On univariate analysis, statistically significant risk factors for illness included kayaking, swimming in the Segama River, swallowing water from the Segama River, and spelunking (Table 2). By multivariate stepwise logistic regression, only swimming in the Segama River was independently associated with illness. Adjusting for the other variables did not alter the relative risk. The attributable risk of river swimming was 36%.

Seventy-six teams, consisting of 4 members per team, competed in Eco-Challenge; at least one member of 62 (82%) of the teams was administered the questionnaire. Of the 51 teams with at least 2 members interviewed, illness and team membership were not significantly associated (data not shown).

Of the 189 athletes contacted, 20 (11%) reported taking doxycycline for prophylaxis of either malaria or leptospirosis. Seventeen of these athletes reported taking a daily dose of doxycycline (100 mg by mouth) throughout the duration of the race; the other three took daily doxycycline, 100 mg by mouth, sporadically throughout the race. Illness developed in 4 (20%) of the 20 athletes who reported taking doxycycline. When the attack rate of those taking doxycycline was compared with the attack rate among those not reporting doxycycline prophylaxis, and adjusted for river swimming, doxycycline usage was protective, although not significantly (relative risk [RR]=0.4, 95% CI=0.1 to 1.1, p=0.1). The preventive efficacy attributable to any doxycycline usage during the race was 55% (95% CI=–0.05% to 95%).

Laboratory Investigation

Clinical symptoms of the athletes and the history of extensive water exposure led to the early consideration of leptospirosis as a diagnosis. In the first few days of the investigation, serum specimens obtained from two of the hospitalized athletes in California were tested at CDC for anti-leptospiral IgM by dot-ELISA dipstick rapid assay; one athlete had a positive test result on a serum specimen obtained 4 days after onset of fever. The second athlete had a negative test result for anti-leptospiral IgM on a serum specimen obtained 4 days after onset of fever but a positive test result on a serum sample obtained 8 days after fever onset. Based on these early results, further diagnostic testing was directed toward leptospirosis.

Of the 80 athletes who met the case definition for illness, serum was collected from 38 (48%). The median duration between onset of fever and the drawing of serum from athletes was 10 days (range 3–34). Of these 38 samples, 26 (68%) tested positive by the dot-ELISA dipstick, the IgM ELISA, or both. No differences in symptoms were found between antibody-positive and antibody-negative patients. Nine additional serum samples were obtained from ill athletes who did not meet the clinical case definition; none of these samples tested positive by rapid assay. Of the 26 samples testing positive by one or both rapid assays, 20 (77%) were subsequently determined to be positive for anti-leptospiral antibodies by MAT. The serogroup associated with the strongest MAT immunoreactivity was Australis.

One culture-confirmed isolate was obtained from an athlete in California. Based on its *rrs* and *secY* gene sequences, this isolate was determined to be most closely related to *Leptospira weilii*, a species found exclusively in Southeast Asia. Serologic analysis showed that the isolate was most likely a new serovar of the Hebdomadis group (8).

Eighteen samples that tested negative for leptospiral antibodies by rapid assay and MAT also tested negative for dengue, typhoid, and scrub typhus antibodies, illnesses that are clinically similar to leptospirosis. These samples included 12 from athletes who met the case definition and 6 from athletes who did not meet the definition. These 18 samples also tested negative for antibodies to Chikungunya, Powassan/tick-borne encephalitis, Sin Nombre virus, Japanese encephalitis, and Snowshoe Hare virus.

Table 2. Risk factors for developing illness, univariate analysis[a]

Exposure	% Ill (n=80)	% Not ill (n=109)	RR	95% CI	p value
Swimming in Segama River	57	28	2.0	1.3 to 3.1	0.002
Swallowing river water	45	21	1.8	1.2 to 2.9	0.008
Kayaking	95	78	3.3	1.2 to 9.0	0.02
Spelunking	87	68	2.2	1.1 to 4.2	0.02

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

Discussion

This outbreak was the first recognized international leptospirosis outbreak associated with the increasingly popular activity of adventure travel. Data from this outbreak investigation suggest that doxycycline may be effective as prophylaxis for leptospirosis in persons with identifiable, short-term exposure to high-risk activities and environments. Preexposure chemoprophylaxis could be increasingly important as more people engage in adventure travel and eco-tourism. In addition, physicians treating returning travelers should consider diseases such as leptospirosis in patients with a history of water exposures. Newer, rapid diagnostic assays may assist physicians in earlier and more accurate diagnosis and, therefore, earlier treatment.

Leptospirosis is a zoonotic disease of worldwide distribution that causes an acute febrile illness; the incubation period is usually 5–14 days but ranges from 2 to 30 days (9). The disease is associated with exposure to water or soil that has been contaminated by a variety of wild and domestic animals, which serve as reservoirs for leptospires and transmit infection by shedding the organisms in their urine (1). The illness is protean. It may be characterized by abrupt onset of fever, chills, myalgias, and headache, and may also include conjunctival suffusion, abdominal pain, vomiting, diarrhea, and skin rashes. Severe leptospirosis may include aseptic meningitis, jaundice, renal failure and hemorrhage; the more severe syndrome characterized by fever, meningismus, and renal and hepatic failure is referred to as Weil's disease. Mild infections can be treated with oral tetracyclines; more severe infections generally require intravenous penicillin (9).

Among the Eco-Challenge athletes, symptoms and exposure history suggested a diagnosis of leptospirosis; this diagnosis was supported early in the investigation by laboratory testing. Our investigation showed a high attack rate in this cohort of athletes (nearly 50% in surveyed athletes). Illness was also severe, with a hospitalization rate of 36% for young, otherwise exceptionally healthy endurance athletes. On September 15, 2000, on the basis of this high attack rate and high proportion of hospitalizations, CDC recommended that all ill athletes be treated with empiric doxycycline and that asymptomatic athletes discuss the possible merits of a single dose of doxycycline with their physicians (10). In addition, asymptomatic athletes were advised to seek medical attention if signs and symptoms consistent with leptospirosis developed.

Although infection or coinfection with other pathogens in ill athletes remains a possibility, testing for antibodies to alternative pathogens commonly causing febrile disease in the tropics was negative in the serum samples tested; most ill athletes were likely infected with leptospires. The known epidemiology of leptospirosis and the epidemiologic data gathered from this investigation suggested a point source for the outbreak associated with exposure to water from the Segama River. The attributable risk of swimming in the river was 36%; those athletes whose illness could not be accounted for by swimming in the river were likely exposed to contaminated water or soil during some other race activity, such as kayaking, trekking, or contact with mud along the river banks. While our study did not find a significant association between swallowing river water and infection, a gastrointestinal route of infection after swallowing contaminated water has also been suggested (11).

Although previously reported in Malaysian Borneo, leptospirosis was not recognized as a cause of a substantial level of illness in Borneo at the time of Eco-Challenge. The known epidemiology and pathophysiology of leptospires suggest that the high attack rate among the athletes was multifactorial. Contact with water from the Segama River was preceded by prolonged treks through jungle vegetation, and all surveyed athletes reported cuts and abrasions (data not shown) that may have predisposed them to a larger inoculum of organisms (12). Another possible factor in the high attack rate may have been rainfall before the race. During Eco-Challenge, rainfall throughout much of the race was heavy, and in the 3 months preceding the event, regional monthly rainfall totals were approximately 250 mm greater than the average for the previous 3 years (National Oceanic and Atmospheric Administration, pers. comm.). Flooding, which has been associated with previous leptospirosis outbreaks, elevates the water table, saturating the soil with leptospires, preventing evaporation of contaminated animal urine, and potentially promoting the survival of leptospires in surface waters (13–16).

Leptospirosis is a relatively common zoonotic disease in the tropics (9); however, it is frequently underdiagnosed because of the nonspecific symptoms associated with infection and the difficulty confirming a diagnosis. MAT is considered the standard diagnostic criterion, but it is technically difficult, not widely available, and involves the use of live leptospires, which presents a hazard to laboratory personnel. A simple, accurate, rapid, and widely available assay for detecting leptospires is needed. In this study, initial screening for leptospires was performed with the Dip-S-Ticks immunoassay, an enzyme-linked dot immunoassay for detecting IgM antibodies; recent evaluations indicate an overall case sensitivity of 98% and specificity of 91% (17,18). The assay has benefits over previous methods of *Leptospira* serodiagnosis because of its ease of use and accessibility, as well as the rapidity of diagnosis; the Dip-S-Ticks assay also has greater sensitivity early in infection than other assays. Three Eco-Challenge athletes tested positive by Dip-S-Ticks but negative by IgM ELISA

(data not shown). Detection of an early nascent immune response remains difficult; this low early sensitivity may partially explain the 50% seronegativity rate in surveyed athletes, given the 10-day median duration between onset of fever and drawing of serum. The inability to obtain MAT confirmation in all specimens that tested positive by rapid assay may similarly be explained by the relatively short interval between onset of fever and the drawing of serum. While MAT reactivity can be seen several days after infection, peak titers are frequently obtained in 2 to 3 weeks; sometimes, however, antibodies do not appear until 3–4 weeks after infection (1). Alternatively, serovars responsible for infection may not have been included in the testing battery used for the serum. The athletes did engage in activities that may have led to multiple exposure risks, and infection with other organisms might have accounted for a proportion of those who tested seronegative for leptospires. We tested some samples for pathogens that also cause acute febrile illness in the tropics; none was detected. However, infection with other organisms remains a possibility.

Due in part to the antigenic heterogeneity among the many different serovars of leptospires, a universally effective leptospirosis vaccine for humans has not been developed; in addition, the duration of immunity with current serovar-specific leptospiral vaccines appears to be short-lived (19). Prevention strategies for leptospirosis have traditionally relied on protective barriers such as wearing rubber boots and gloves and avoiding high-risk areas. However, several studies of persons from areas endemic for leptospirosis and of military recruits with no known prior exposure to leptospires have shown that doxycycline given before or shortly after exposure can reduce illness and death caused by leptospirosis (2–4). In our study, athletes who took doxycycline for malaria prophylaxis were less likely to become ill; although this finding did not reach statistical significance, we may not have had sufficient data to detect significance. Persons who travel to areas where leptospirosis is endemic or epidemic and who participate in high-risk exposure activities involving prolonged water exposure may be at increased risk for leptospirosis (11,14,20,21) and may benefit from chemoprophylaxis. Until additional efficacy data are available, persons at high risk for leptospirosis should consider chemoprophylaxis with doxycycline, 200 mg by mouth per week, beginning 1–2 days before exposure and continuing for the duration of suspected exposure. The role of other antimicrobial agents is not clear. Further randomized, controlled studies assessing the utility and efficacy of doxycycline and other antimicrobial agents for the prophylaxis of leptospirosis are needed.

More people are participating in exotic travel, adventure sports, and eco-tourism, and as a result, the potential for contact with pathogens less common in the temperate, industrialized world, such as *Leptospira,* is likely to increase. Outbreaks of leptospirosis have previously been documented in athletes participating in such activities as kayaking (14), swimming (20), and triathlons (11). Recently, leptospirosis in endurance athletes has been the subject of several reports (22–24). In addition, previous outbreaks of infectious disease attributable to other unusual pathogens have been documented in adventure travelers (25,26). Since leptospires are a common cause of febrile illness in the tropics, physicians should evaluate travelers with febrile illness returning from these areas for this and other emerging infections.

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Community Reaction to Bioterrorism: Prospective Study of Simulated Outbreak

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To assess community needs for public information during a bioterrorism-related crisis, we simulated an intentional Rift Valley fever outbreak in a community in the southern part of the United States. We videotaped a series of simulated print and television "news reports" over a fictional 9-day crisis period and invited various groups (e.g., first-responders and their spouses or partners, journalists) within the selected community to view the videotape and respond to questions about their reactions. All responses were given anonymously. First-responders and their spouses or partners varied in their reactions about how the crisis affected family harmony and job performance. Local journalists exhibited considerable personal fear and confusion. All groups demanded, and put more trust in, information from local sources. These findings may have implications for risk communication during bioterrorism-related outbreaks.

Human behavior during disasters (e.g., hurricanes, fires, mass shootings, airplane crashes) has been studied by historians as well as behavioral and social scientists, and disaster management teams make assumptions on the basis of these studies (1–11). However, with bioterrorism (intentional release of biological, chemical, or radiologic agents), the standard sensory cues (location, beginning and end of crisis) are not available; therefore, a different "emotional valence" may be involved. The standard models used as predictors of human behavior during crises may not be adequate. We simulated a bioterrorism-related outbreak in a U.S. community to examine (prospectively) the community's reaction to the crisis and assess the need for public information.

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Methods

We simulated the intentional aerosolized release of Rift Valley fever virus (RVFV) in a semirural community (population 300,000) in the southern part of the United States. The community was selected because its mosquito population could support transmission of RVFV. We videotaped a series of simulated print and television (local, network, and cable) "news reports" over a fictional 9-day crisis period. The 83-minute videotape told the story of the intentional disease outbreak. We invited four groups (medical first-responders, medical first-responder spouses or partners, journalists, and others) within the selected community to view the videotape and answer questions about their reactions. These four groups knew that the outbreak was fictional. We then tabulated and analyzed the responses.

The Video

The story of the simulated outbreak unfolded in a series of video reports from federal and local governments and the news media (Appendix 1, online only, available from: URL: http://www.cdc.gov/ncidod/EID/vol9no6/02-0769_app1.htm). Health agency news bulletins were provided by the Centers for Disease Control and Prevention; news reports by television reporters or news anchors; and community reports by local officials, including the mayor. The reports began with recognition in the community of an unusual infection affecting humans and certain farm animals and continued during the next 9 days with an epidemiologic investigation and the identification by federal authorities of intentional release of RVFV. Reports included a detailed press conference by federal health authorities describing routes of transmission, prevention measures, signs and symptoms of infection, and medical management of the disease. The news conference, held in the state capital the day after the presence of RVFV infection in the United States was announced, was immediately followed by a panel discussion (by nongovernment experts) on RVFV. Differences of opinion on clinical, epidemiologic, and biological issues among RVFV experts were reported.

Confusion arose in the community over disease management (e.g., the effectiveness of the antiviral drug ribavirin, the need for RVFV vaccine, and who should receive the vaccine) and over the potential for infected persons to serve as reservoirs and carriers of the virus elsewhere during the few days when viral titers are especially high. Governors of adjoining states questioned the adequacy of mosquito-control and animal quarantine measures, given the lack of a control model for the spread of RVFV infection in industrialized countries. Although official quarantine measures were not taken, final video reports showed a gradual de facto isolation of the city.

The Questionnaire

The questionnaire (Appendix 2, online only, available from: URL: http://www.cdc.gov/ncidod/EID/vol9no6/02-0769_app2.htm), which included multiple-choice, open-ended questions, and opportunities for additional comments, was distributed to all participants. Questions addressed job abandonment, quarantine compliance, demand for drugs and vaccine, information requirements, and other issues of community interest. Six sets of questions were posed to the participants during the video presentation. Set 1 was given after a disease of unknown etiology affecting humans and some farm animals was recognized in the community. These questions focused on willingness to remain at work, the types and sources of information that influenced the decision to work or not work, and actions regarding families and loved ones. Set 2 was given after the disease was identified as RVFV infection, federal health authorities briefed the public about this infection at a press conference, and a panel of nongovernment experts discussed the disease on television. These questions tested the participants' understanding of RVFV routes of transmission and preventive measures and the participants' satisfaction with information from government and nongovernment sources. Sets 3 and 4 followed a period of growing confusion and anxiety caused by changing and sometimes conflicting "authoritative" statements and tested participants' requests for medication, including ribavirin (Set 3), and for RVFV vaccine (Set 4). Set 5, given after the participants learned that the outbreak of RVFV was intentional, reassessed decisions and actions regarding job and family concerns and information needed to make these decisions. Set 6 followed a period of increasing anxiety over a now-confirmed bioterrorism-related outbreak that could spread to humans and cattle in the state and in adjoining states, over the ability of the government to stop the spread of the infection, and over the de facto isolation of the community. These questions surveyed participants' reactions to rumors of possible quarantine and to sources of information deemed reliable and influential in decision making now that the threat had become more complicated, personal, and disruptive.

Participants

Four study groups, totaling 153 community residents, were formed. A goal of at least 30 participants per group was dictated by budgetary factors. The number of candidates contacted to assemble groups of at least 30 participants followed guidelines from marketing study groups (Harwell Productions, pers. comm.). One hundred thirty-eight medical first-responders (responders) were invited to participate in one study group; 58 responded to our invitation, 45 registered, and 38 attended. Reflecting the make-up of medical first-responders in the community, one third of the group's participants were fire department emergency medical services personnel, and two thirds were emergency department personnel (nurses, physicians, technicians) from the area's major medical centers. Eighty-three spouses or partners of responders (hereafter termed spouses) were invited to form a second study group; 47 responded, 44 registered, and 32 attended. Fifty-seven members of the local print and TV news media (hereafter, termed the media) were invited to a third study group; 50 responded, 42 registered, and 34 attended. Three hundred fifty invitations were sent to rank-and-file residents of the community (hereafter termed residents) to form a final study group. Twenty-three invitations were returned by the U.S. Postal Service as undeliverable; 73 responded, 46 registered, and 47 attended (1 registered participant failed to appear, but 2 invitees who had not registered arrived and participated).

Names of persons invited to participate in the first three groups were drawn blindly from rosters of eligible candidates prepared by their employers. Rosters of eligible candidates for the residents category were prepared from census tract data and cross-indexed telephone books (Table).

Sex and age distributions within the four study groups were representative of the population segments from which their members were drawn, as were the educational levels of the responder, spouse, and media groups. The educational distribution of residents was not representative of the metropolitan area; those who chose to participate in this group had more formal education than area residents in general. According to 2000 census data for this area, 24.2% of the population have no high school diploma or equivalency, 30.2% are high school graduates, 22.4% have some college education, 12.7% have either an associate or full college degree, and 6.1% have graduate or professional degrees. We asked all participants if they had family members and loved ones in the area to assess potential conflict in job loyalty versus safety of one's family and loved ones during a disaster. The media group was unique

Table. Study group characteristics, simulated bioterrorism incident

	Responders (N=39) no. (%)	Spouses (N=32) no. (%)	Media (N=34) no. (%)	Residents (N=48) no. (%)
Sex				
Male	24 (61.5)	12 (37.5)	21 (61.8)	22 (45.8)
Female	15 (38.5)	20 (62.5)	13 (38.2)	26 (54.2)
Age				
18–26	7 (17.9)	6 (18.8)	4 (11.8)	4 (8.3)
27–50	30 (76.9)	22 (68.8)	23 (67.6)	19 (39.6)
51–65	2 (5.1)	3 (9.4)	6 (17.6)	11 (22.9)
>65		1 (3.1)	1 (2.9)	14 (29.2)
Family members in area				
Yes	38 (97.4)	31 (96.9)	22 (64.7)	44 (91.7)
No			12 (35.3)	4 (8.3)
No response	1 (2.6)	1 (3.1)		
Education				
Not high school graduate		1 (3.1)		1 (2.1)
High school diploma/ equivalency	7 (17.9)	8 (25.0)		6 (12.5)
Some college	15 (38.5)	18 (56.3)	5 (14.7)	16 (33.3)
College degree	13 (33.3)	3 (9.4)	25 (73.5)	16 (33.3)
Post-graduate education	4 (10.3)	1 (3.1)	3 (8.8)	7 (14.6)
No response		1 (3.1)		
Other			1 (3.0)	2 (4.2)

in the large number of its members who answered no to this question.

Results

When asked about the transmission of RVFV, 30% to 35% of all group participants knew that the virus is transmitted by mosquitoes and not from person to person. RVFV transmission information was given by federal authorities at the televised press conference, as well as by academic experts interviewed on television after the press conference. The largest group who thought the risk for person-to-person transmission was "considerable" was responders. None of the participants was satisfied to receive information from federal authorities only. Most participants wanted additional information from local public health authorities, and 48% to 75% wanted information from both government and nongovernment sources.

In all four groups, participants who expressed interest in nonmedically indicated antibiotic or ribavirin treatment were in the minority. The largest minority to demand such medications was in the group of spouses; however, the demanded medication was primarily for their first-responder companions, not for themselves. In all four groups, approximately 50% of participants said that they would compete for RVFV vaccine for themselves and their families; the largest demand came from the media. When asked if they would demand vaccine for themselves as a quid pro quo for remaining on the job during the crisis, 26% of responders said they would, and 21% said they were uncertain. Sixty-three percent of spouses said they would want their mates to demand vaccine.

Reacting to rumors of quarantine (not to an official quarantine announcement), 59% of media and 75% of residents said they would comply and not try to leave; 6% of residents and 13% of spouses said they would try to leave regardless of consequences; 4% of residents and 15% of media said they would obey but try to leave if necessary. In all groups, most participants stated that their willingness to comply would increase if they were assured that quarantine was absolutely necessary and that it would work. In all groups, a majority faulted federal authorities for holding the joint press conference in the state capital, rather than at the scene of the outbreak 250 miles away.

Early in the simulated outbreak, before the disease was identified and its implications were known, pluralities in all groups wanted health information from local public health authorities. Responders wanted the information delivered within the chain of command at work; ranking second as the desired source of this information for all groups, except responders, was the private physician. After the disease was identified and terrorism was determined the cause, no single source of information at any level was chosen as desirable by any majority. Small pluralities in each group chose as the most reliable source of information "the head of the federal team working at the outbreak site," "the President," "a physician from a federal agency," or "other." When asked whom the participants considered most influential in decisions they would make about work, family, and themselves, once again no single authority figure emerged as the majority's selection in any group. Selections with small pluralities were "family and loved ones," "the President," and "the head of the federal

team working at the outbreak site." Half the participants in all groups chose "other." Within "other," 34% of the participants chose another federal medical or nonmedical official, 4% chose state officials, 5% chose national (not local) media, 11% said they did not know at that time in the outbreak who would be most influential in their personal decision making, and 47% chose local leaders, including government and nongovernment officials.

The media indicated they would turn to a variety of local sources for their assignments. Most of these sources were professors in the local medical school, but other sources included the reporters' personal physicians and veterinarians.

Most participants in all four study groups indicated they would remain on the job throughout the crisis. Initially, when the outbreak of an as-yet-unknown disease in the area was recognized, 97% of responders, 94% of media, and 77% of residents said they would remain at work; 11% of residents opted not to work, and the remainder said they were students, unemployed, or retired. Ninety-one percent of spouses said they would encourage their mates to continue working.

After the disease was identified and the outbreak was recognized as an act of bioterrorism, 95%, 71%, and 65% of responders, media, and residents, respectively, said they would continue working, and 78% of spouses said they would want their mates to remain on the job. In all groups, most participants said they would continue to work, provided that they received information about medical issues (particularly transmission and prevention), their work sites were adequately protected, and the community were unlikely to be exposed to another act of bioterrorism. Seventy-seven percent of media said that if their work put them at risk, they would expect their employers to provide protective measures (from insecticides to vaccine) and necessary medication and treatment.

Just before conclusion of the video, responders and spouses were asked how important it was to reach agreement with their partners on whether to stay at work, seek medicines, and send family members out of town. Twenty-six percent of responders said that concurrence would be essential, whereas 53% of spouses thought such agreement was essential.

Discussion and Conclusions

The study was based on a simulated outbreak; therefore, the participants' reactions were to a simulated, not an actual, crisis. For budgetary reasons, we could not recruit and compensate sufficient numbers of community residents to provide a statistical sample of the populations from which they were drawn; therefore, as with some forms of market research, participants' answers to questions reflect their opinions and not those of their peers. Sample bias may have resulted from the absence of persons invited to participate who declined to do so; this may be especially important among residents, whose undereducated members were underrepresented. The choice of RVFV as the disseminated agent may have muted the responses of the participants because it has a relatively low death rate (1% to 10%) and does not have the high "fear factor" of some other diseases such as smallpox. Finally, the community we chose has its own customs, traditions, and ways of coping with a crisis that may not be shared by communities elsewhere.

Some participant reactions in this simulated-outbreak study are of particular interest. For example, disagreements between responders and spouses over reporting for duty during the crisis and demands for vaccine as quid pro quo for staying on the job could influence staffing levels and responder job performance in an actual bioterrorism-related outbreak. Risk communication messages may need to be crafted, tailored to the needs and concerns of first-responders and families, and delivered separately.

Journalists are key participants in risk communication (12–16), yet in this study, the media exhibited more fear than any group other than spouses, made high demands for vaccine, had the poorest understanding of medical issues associated with RVFV, and were most likely to stay away from work after terrorism was recognized. Had this been an actual bioterrorism-related outbreak, the media might not have served effectively as conduits of information to the public because they had not been adequately educated to eliminate confusion and dispel fear about their personal safety.

Participants in this study were not unique in their wariness of sole-source information, however authoritative or expert (17–20). Members of the actual community in which the simulated bioterrorism-related outbreak occurred wanted information from varied sources, even if the sources they mentioned differed in quality and reliability. Their reactions suggest that bioterrorism training should include information management for risk communicators and public affairs officers who have the responsibility of providing timely and accurate information to dispel the "fog" of rumors and misinformation present during the aftermath of an intentional disease outbreak. Journalists and other media specialists should participate actively in scenarios and other similar exercises to gain insight into the complexity of information management in a bioterrorism-related crisis.

As the simulated outbreak became more complicated and personally threatening, participants indicated a preference for information from local government and nongovernment sources or from federal officials at the outbreak site. Recognized, respected community leaders (e.g., private physicians, government and nongovernment offi-

cials) are likely to provide guidance in a bioterrorism-related crisis, just as they do in other crises. Training in the strategy and tactics of risk communication (21–24) should be expanded to include them. In an actual bioterrorism-related outbreak, these local leaders, supported by federal health authorities, should take the lead in communicating with local residents.

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Instructions for Emerging Infectious Diseases Authors

Research

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").

Severe Acute Respiratory Syndrome (SARS) in Singapore: Clinical Features of Index Patient and Initial Contacts

Li-Yang Hsu,* Cheng-Chuan Lee,* Justin A. Green,* Brenda Ang,* Nicholas I. Paton,* Lawrence Lee,* Jorge S. Villacian,* Poh-Lian Lim,* Arul Earnest,* and Yee-Sin Leo*

Severe acute respiratory syndrome (SARS) is an emerging viral infectious disease. One of the largest outbreaks of SARS to date began in Singapore in March 2003. We describe the clinical, laboratory, and radiologic features of the index patient and the patient's initial contacts affected with probable SARS.

Severe acute respiratory syndrome (SARS), an atypical pneumonia characterized by high rate of transmission to healthcare workers (1), began in Guangdong Province, China, in November 2002. One of the largest SARS outbreaks to date began in Singapore in mid-March 2003 and was traced to a traveler returning from Hong Kong.

According to the World Health Organization, a suspected case of SARS is defined as documented fever (temperature >38°C), lower respiratory tract symptoms, and contact with a person believed to have had SARS or history of travel to an area of documented transmission. A probable case is a suspected case with chest radiographic findings of pneumonia, acute respiratory distress syndrome (ARDS), or an unexplained respiratory illness resulting in death, with autopsy findings of ARDS without identifiable cause (2). We describe the clinical features of the index patient in Singapore and the patient's initial group of contacts affected with probable SARS.

The Index Case

The index case of SARS in Singapore ocurred in a previously healthy 23-year-old woman of Chinese ethnicity who had stayed on the 9th floor of a hotel during a vacation to Hong Kong, February 20–25, 2003. A physician from southern China who stayed on the same floor of the hotel during this period is believed to have been the source of infection for this index patient and the index patients of outbreaks in Vietnam and Canada.

Fever and headache developed in the patient on February 25 and a dry cough on February 28. She was admitted to Tan Tock Seng Hospital, Singapore, on March 1. On admission she had oral temperature of 37.6°C and was lethargic. The chest was clear to auscultation. The remainder of her physical examination was normal. The total leukocyte count (2.7×10^9/L), lymphocyte count (0.9×10^9/L), and platelet count (102×10^9/L) were reduced below normal laboratory ranges. Electrolytes and liver biochemistry results were normal. The chest x-ray showed patchy consolidation of both upper and lower lobes of her right lung (Figure 1a). Blood cultures were sterile, and tests for urinary *Legionella* antigen, particle agglutination test for *Mycoplasma pneumoniae* antibodies, and complement fixation test for *Chlamydia* antibodies were negative. Immunofluorescence performed on nasopharyngeal aspirates for viral antigens of influenza virus A and B, parainfluenza virus, respiratory syncytial virus, and adenovirus was negative.

Intravenous levofloxacin, 500 mg once a day, was administered, but the patient's temperature continued to spike up to 40°C, and the cough persisted. On day 5 of hospitalization, she became breathless and required supplemental oxygen. Sequential chest x-rays showed progressive, extensive involvement of the right lung, with new infiltrates appearing on the left (Figure 1c). Liver enzymes

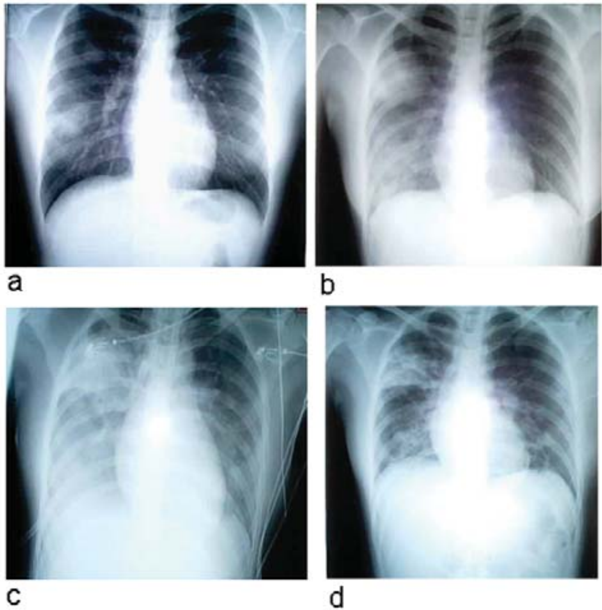

Figure 1. Chest radiographs of index patient with severe acute respiratory syndrome (SARS). a, day 5 of symptoms; b, day 10; c, day 13; d, day 15.

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became elevated, with an ALT of 200 U/L (7–36 U/L) and AST of 208 U/L (15–33 U/L); serum lactate dehydrogenase (LDH) levels rose to 1518 U/L (200–500 U/L). Intravenous vancomycin (1 g twice a day) and oral oseltamivir (75 mg twice a day) were added to the regimen. Nine days after admission, the patient began to improve clinically, the laboratory abnormalities returned towards normal, and the chest x-ray abnormalities stabilized and resolved. The patient has remained well.

Electron microscopy of the specimens obtained by nasopharyngeal aspiration on day 7 of hospitalization showed viral particles of <100 nm with widely spaced, club-shaped surface projections characteristic of coronaviruses.

Clinical Features of Contact Cases

When the index patient was seen in early March, the clinical features and highly infectious nature of SARS were not known. For the first 6 days of hospitalization, the patient was in a general ward, without barrier infection control measures. One of eight physicians who attended her became infected, as did 9 of approximately 30 nursing staff. SARS also developed in 1 of 12 patients in adjacent beds during her hospitalization and 9 of approximately 30 family members and friends who visited her during this time. Nineteen of these 20 patients were admitted to our hospital for treatment and isolation (1 was treated outside Singapore), and we recorded prospectively the clinical features of their illnesses with a standardized data collection form. In addition to demographic data, this form elicited information on occupation, date(s) of exposure to suspected cases, travel history after February 20, dates of onset of various symptoms, results of blood tests, and chest radiographic findings.

The demographic profiles of the index and 19 contact cases are shown (Table 1). An epidemic curve of the index and contact cases is shown in Figure 2. Because most healthcare staff in our hospital are women, a high proportion of the case-patients (75%) were female. The median age of patients was 28 years. All were previously healthy, except one who had diabetes mellitus and end-stage renal failure and one who had a history of childhood asthma. One patient was a smoker. For seven patients who only had one exposure to the index patient, the median incubation period was 4 days (estimated range 2–8 days). For those with multiple exposures (13 patients), median incubation period was either 7 days (range 4–12 days, calculated from day 1 of exposure), or 5 days (range 3–9 days, calculated from midpoint of exposure period). The median period from onset of symptoms to admission was 6 days (range 0–9 days)

At admission, all patients had fever, sometimes accompanied by myalgia and headache (Table 2). Other symptoms, including dry cough, developed 2–4 days after onset of fever. Shortness of breath (when present) generally manifested in week 2 of illness. Apart from elevated temperature, results of physical examination were generally normal. The paucity of lung findings was often in striking contrast to the florid chest radiographic changes.

Table 1. Demographic description of patients with severe acute respiratory syndrome, Singapore

Demographics	No.
No. of men (%)[a]	5 (25)
No. of healthcare workers (%)[a]	9 (45)
Median age in years (range)	28 (19–73)
Median days from onset of symptoms to admission (range)	6.0 (0–9)

[a]N=20.

Laboratory investigations at admission are shown (Table 3). Lymphopenia, as defined by a cell count of <1.5 x 10^9/L, was present in 18 of 20 patients. Other common laboratory abnormalities included leukopenia, thrombocytopenia, elevated LDH, mild hyponatremia, mild hypokalemia, and raised hepatic transaminases.

Abnormal chest radiographs were seen in 14 patients at admission. Abnormalities developed in the remaining six patients by day 11 of illness. The most common pattern noted initially was interstitial infiltrates at the base of the right lung (11/20 patients). Right upper lobe infiltrates were seen in four patients, and involvement of both right upper and lower lobes was seen in three. One patient each had left lower lobe and bibasal infiltrates. Radiographic abnormalities rapidly progressed in all but one patient, and an ARDS-type picture developed in six patients, who subsequently required mechanical ventilation (Figures 1, 3).

Results of routine microbiologic cultures, serologic tests, and rapid antigen tests were universally negative. However, viral particles characteristic of coronavirus were found on electron microscopy examination of nasopharyngeal aspirates in 4 of 10 patients.

The clinical course of the illness is shown (Table 4). In 11 patients, oxygen saturation fell below 95%, and supple-

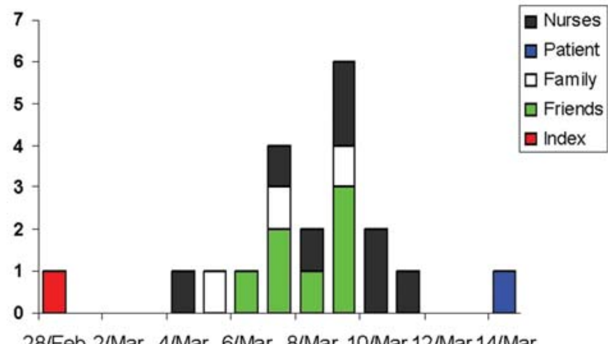

Figure 2. Index and contact cases of severe acute respiratory syndrome (SARS), by date of symptom onset.

Table 2. Clinical features of severe acute respiratory syndrome

Symptom	No. (%) (N=20)
Fever	20 (100)
Dry cough	15 (75)
Myalgia	9 (45)
Malaise	9 (45)
Anorexia	9 (45)
Shortness of breath	8 (40)
Nausea/ vomiting	7 (35)
Sore throat	5 (25)
Diarrhea	5 (25)
Headache	4 (20)
Chills and rigors	3 (15)
Rhinorrhea	3 (15)

mental oxygen was instituted. Of these patients, six subsequently required mechanical ventilation for worsening respiratory failure. Clinical deterioration generally occurred in week 2 of illness.

All patients were initially treated with either levofloxacin or a combination of intravenous ceftriaxone and a macrolide once x-ray abnormalities were observed. Oseltamivir, 75 mg twice a day, and oral ribavirin, 20 mg/kg body weight three times a day, were prescribed to 6 and 14 patients, respectively. These antiviral drugs were started late in the course of illness: Most were prescribed from day 10 to day 14 of symptoms. Five patients in the intensive care unit were given corticosteroids (intravenous hydrocortisone, 100 mg every 6 hours, or intravenous methylprednisolone, 120 mg once a day).

Patients with ARDS were supported aggressively with intubation and mechanical, pressure-controlled ventilation with high positive end-expiratory pressures. Despite these and other supportive measures, three patients died of progressive respiratory failure.

Fever and laboratory and radiologic abnormalities resolved beginning from day 10 of illness. Patients with milder disease improved earlier. Of six patients who required mechanical ventilation, three died, and two improved sufficiently to be extubated. The last patient remains critically ill at the time of writing.

Conclusions

Initial clinical features of SARS are nonspecific. Dry cough is common, although other symptoms of upper respiratory tract infection are unusual. Physical signs on chest examination are minimal, and chest radiograph may be normal on week 1 of illness. Laboratory tests often show lymphopenia, mild thrombocytopenia, and elevated liver enzymes. Therefore, in early stages, SARS may be hard to differentiate from other viral infections, and diagnostic delays may contribute to the spread of the epidemic. Early diagnosis relies on known history of potential exposure to SARS. Clinicians must maintain a high index of suspicion and be familiar with the rapidly changing epidemiology of this infection. Early diagnosis minimizes transmission.

In this initial cohort, radiologic changes eventually developed in all contacts with fever. However, the extent of chest x-ray changes and respiratory failure varied widely, and subsequent observations suggest a milder form of illness in which radiologic changes are not apparent. Whether asymptomatic infection can occur is unknown, but a more comprehensive description of the spectrum of clinical illness will only be possible when a diagnostic test is available. To this end, we are collecting serum from healthcare staff exposed to SARS patients.

The index patient infected at least 20 others over a period of several days. These cases were probably secondary rather than tertiary because the dates of symptom onset were very close and little interaction occurred between contacts. Patient 20 had a relatively late onset, but she was discharged from our hospital by March 6 and had no subsequent contact with other case-patients. Clearly, this infection is highly transmissible from person to person, and healthcare workers are particularly at risk (3–5). The

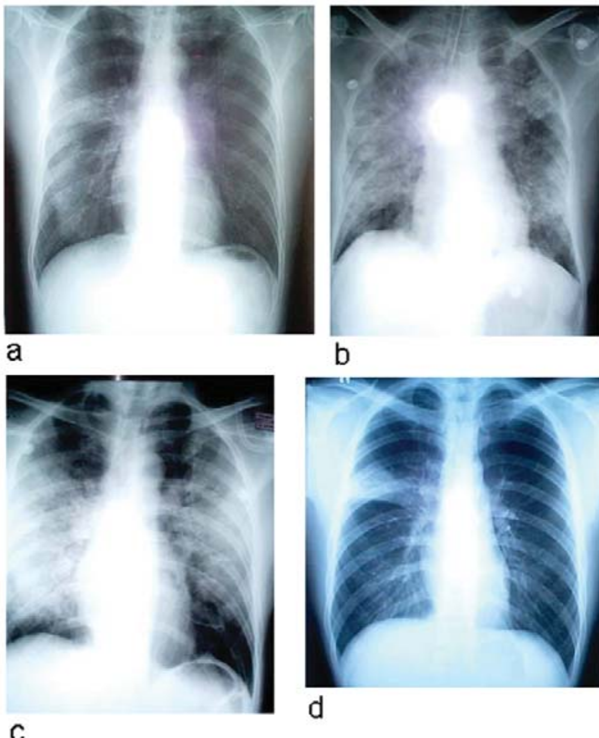

Figure 3. Chest radiographs of two patients with severe acute respiratory syndrome (SARS). a–c: radiographs of patient 5 showing progression of changes. a, day 8 of symptoms; b, day 13 of symptoms, c, day 14 of symptoms. He died on day 19 of this illness. d, chest radiograph, taken on day 8 of symptoms, of patient 12, with right upper lobe infiltrates resembling pulmonary tuberculosis (TB) but laryngeal swab cultures for TB were negative.

Table 3. Summary of severe acute respiratory syndrome signs and laboratory tests done at admission, Singapore, 2003

Test	Mean (SD)	Median (range)
Temperature (°C)	38.3 (0.9)	38.4 (37–40)
Oxygen saturation (%)	97.9 (1.8)	98 (92–100)
Leukocytes (4–10 x10^9/L)	4.8 (2.2)	4.2 (2.5–10.6)
Lymphocytes (1.5–4.3 x10^9/L)	0.9 (0.4)	0.7 (0.5–1.7)
Platelet count (160–390 x 10^9/L)	159 (48.3)	151 (98–272)
Alanine aminotransferase (7–36 U/L)	40.6 (78)	17 (7–355)
Lactate dehydrogenase (200–500 U/L)	532.2 (260)	432 (306–1142)
Albumin (40–50 g/L)	38.6 (5.1)	39 (25–46)
Globulin (25–40 g/L)	33.9 (4.6)	35 (25–45)
Urea (2–7.5 mmol/L)[a]	3.2 (1.5)	2.9 (1.9–8.5)
Creatinine (25–100 umol/L)[a]	65.4 (12.4)	65 (39–88)
Sodium (135–145 mmol/L)[a]	134.9 (2.8)	135 (131–141)
Potassium (3.5–5.0 mmol/L)[a]	3.5 (0.3)	3.6 (2.8–4)

[a]The laboratory results of patient 20, who had end-stage renal failure, were not included in this table.

precise routes of transmission in a healthcare setting need to be defined, although the predominance of right lower lobe findings on chest radiography suggests that droplet or airborne transmission is involved. However, were airborne transmission involved, we would have seen a much greater number of cases with weaker contact links to the index patient. We did not see any further transmission from this index patient after we implemented strict infection control measures involving use of N95 masks, gown, gloves, and handwashing before and after patient contact. Which components of this approach are responsible for the decrease in transmission is unclear.

A coronavirus was identified on electron microscopy of nasopharyngeal aspirates in our index patient and several of her contacts and is therefore likely to be responsible for this outbreak. This finding concurs with those in SARS outbreaks in other countries (6). Coronaviruses are widespread in the animal kingdom, and human coronaviruses are one of the main causes of the common cold. These viruses are also an important cause of pneumonia in military recruits (7). The aggressive nature of this disease suggests a new variant coronavirus; preliminary studies (3–5) confirm this hypothesis.

No effective treatment is known for this infection. We did not observe any obvious response to antibiotic therapy. However, until a specific diagnostic test is widely available, physicians should consider the use of empiric antibiotic therapy, including for atypical organisms in cases of probable SARS in which bacterial pneumonia cannot be definitely excluded.

Table 4. Clinical course of severe acute respiratory syndrome (days after onset of symptoms) occurring up to April 10, 2003

Patient	Sex[a]	Age	Date of onset	Admission to hospital	First abnormal chest x-ray	Supplemental oxygen requirement	Mechanical ventilation	Fever resolved	Radiologic improvement	(Death)/ medically fit for discharge
1 (Index)	F	23	February 25	5	5	13	Not required	18	15	20
2	F	45	March 4	9	9	13	13	22	20	Hospitalized
3	F	46	March 5	6	6	11	13	Not seen	Not seen	(34)
4	M	25	March 6	9	9	Not required	Not required	12	14	19
5	M	50	March 7	8	8	8	10	Not seen	Not seen	(19)
6	F	27	March 7	5	8	Not required	Not required	16	16	21
7	F	23	March 7	5	5	Not required	Not required	10	13	15
8	M	40	March 7	6	6	8	10	Not seen	Not seen	(20)
9	F	28	March 8	7	9	Not required	Not required	11	12	13
10	F	26	March 8	7	7	11	Not required	14	14	17
11	M	27	March 9	8	8	Not required	Not required	12	14	17
12	M	39	March 9	7	11	Not required	Not required	13	15	18
13	F	19	March 9	7	7	13	Not required	20	17	23
14	F	73	March 9	7	7	10	11	12	18	20
15	F	23	March 9	6	6	11	Not required	16	16	19
16	F	35	March 9	0	9	Not required	Not required	11	12	13
17	F	22	March 10	7	11	Not required	Not required	13	14	17
18	F	30	March 10	5	9	Not required	Not required	11	14	15
19	F	22	March1 1	4	4	8	Not required	10	13	14
20	F	42	March 14	4	4	4	5	9	10	Hospitalized

[a]F, female; M, male.

Steroids were prescribed for five of six patients who had ARDS and were on mechanical ventilation. No perceived benefits accrued from this practice, although smaller doses were used than in Hong Kong (4,5).

A number of our patients received ribavirin, a broad-spectrum antiviral drug with known activity against some RNA viruses. However, we did not observe any obvious response to this drug, and several patients deteriorated in spite of its use. In contrast, a number of patients (including our index patient) recovered without use of ribavirin. Although some anecdotal reports of efficacy of ribavirin (when used in conjunction with steroids) exist (4,5), its efficacy is hard to judge for a condition that has potential for spontaneous recovery. Evidence from randomized controlled trials is needed before the use of ribavirin can be advocated for routine use in SARS patients.

Acknowledgments

We thank the medical officers and staff of Tan Tock Seng Hospital, Singapore, for their courage and dedication in caring for SARS patients.

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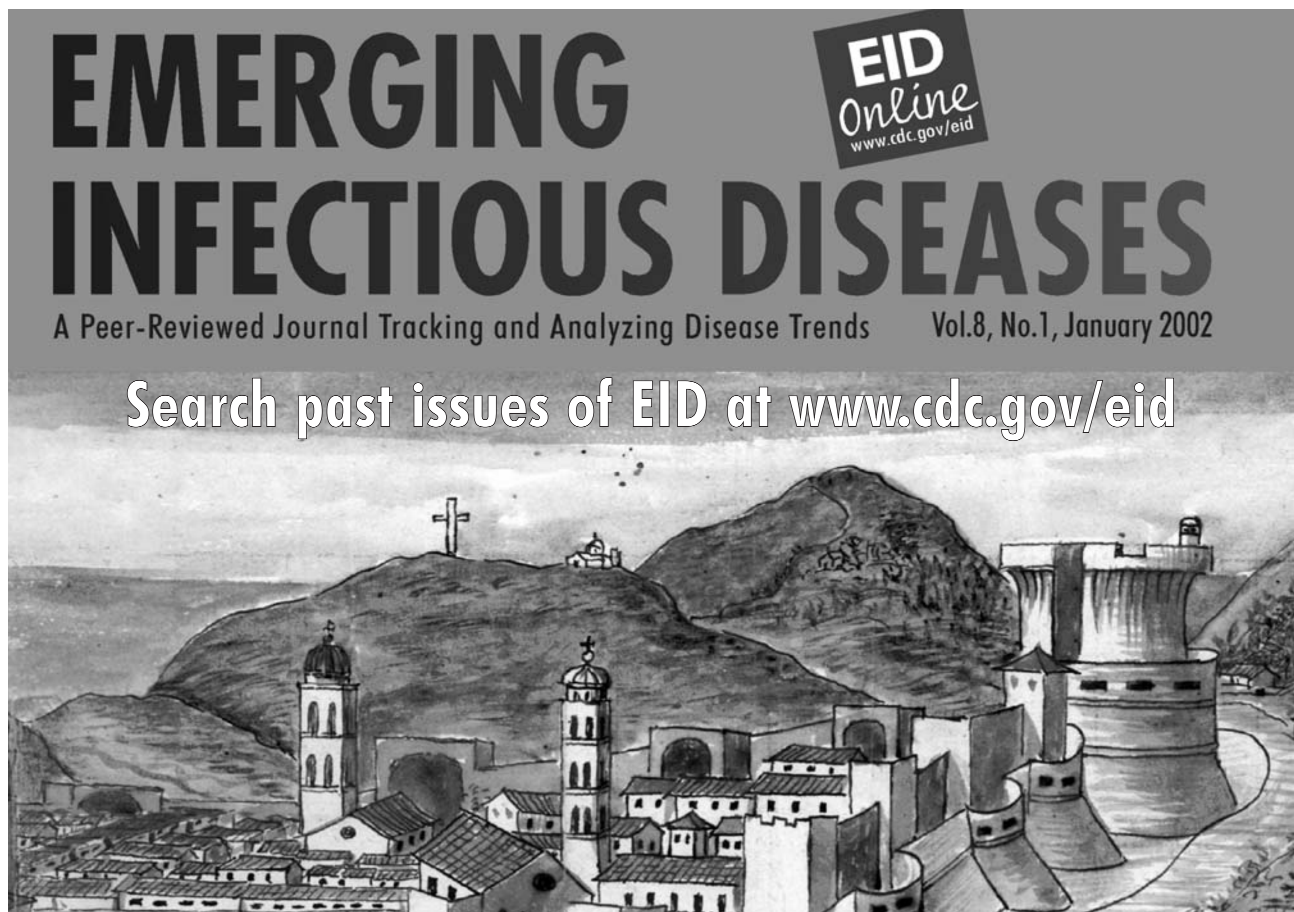

Control Measures for Severe Acute Respiratory Syndrome (SARS) in Taiwan

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As of April 14, 2003, Taiwan had had 23 probable cases of severe acute respiratory syndrome (SARS), 19 of which were imported. Taiwan isolated all 23 patients in negative-pressure rooms; extensive personal protective equipment was used for healthcare workers and visitors. For the first 6 weeks of the SARS outbreak, recognized spread was limited to one healthcare worker and three household contacts.

The global spread of severe acute respiratory syndrome (SARS) has proceeded with unprecedented speed, overwhelming many hospitals and some public health systems in a matter of weeks. As of April 14, 2003, a total of 3,169 cases had been reported from more than 20 countries. In many locations, the introduction of the disease by ill travelers has soon been followed by spread to healthcare workers and household contacts. In the most mature outbreaks, in Hong Kong and Hanoi, 46% and 63% of cases, respectively, were reported in healthcare workers, and hospital spread has also characterized the larger outbreaks in Singapore and Toronto (1,2).

Taiwan, with its close proximity to the epicenters of severe acute respiratory syndrome (SARS) in Guangdong Province and Hong Kong and its extensive business and cultural ties, has heavy travel volume from the most affected areas. The first probable SARS case-patient in Taiwan returned from Guangdong and Hong Kong early in the global outbreak, on February 21, 2003, and a series of other importations have been documented since that time. Factors that contribute to spread of infection in a given location are not well understood but may include not only the number of coronavirus-infected persons but also whether any of these persons are particularly infectious, whether they are identified early in their illness, and how effectively they are isolated. To contribute to discussions on how to effectively prevent transmission, we believe reporting the early experience with limited spread of the disease in Taiwan, along with a thorough description of the control measures taken, is important.

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Epidemiology of SARS in Taiwan

The first recognized SARS patient in Taiwan was in a 54-year-old businessman who traveled to Guangdong Province, China, on February 5, 2003, and returned to Taipei by way of Hong Kong on February 21. On February 25, fever and myalgia, and later a dry cough, developed, but he was not hospitalized until March 8. Several hours after admission, he was intubated and required mechanical ventilation for 13 days. During the initial hospitalization, he was cared for in a single intensive care unit (ICU) room by healthcare workers who used standard nursing (universal) precautions. When pneumonia was diagnosed in the patient's wife on the morning of March 14, both patients were placed in isolation rooms; by the afternoon they were isolated in ICU negative-pressure rooms with full precautions, as described below. Fever developed in their son on March 17, followed by cough on March 20; he was hospitalized in a negative-pressure isolation room on March 21. The wife and son were exposed during the period before full protective measures were in place, and SARS developed in both. Both required mechanical ventilation. The illnesses in the wife and son were confirmed by reverse-transcription–polymerase chain reaction (RT-PCR) testing to be associated with the novel SARS coronavirus (3–5).

As of April 14, 23 persons in Taiwan met the World Health Organization (WHO) criteria for a probable case of SARS. Of these, eight had SARS-associated coronavirus identified in throat swabs by PCR. An additional 120 reports of possible case-patients with compatible travel or contact were investigated, and 13 remained under investigation. Of the probable case-patients, 19 (83%) reported travel to mainland China and Hong Kong in the 10 days before illness onset, and 4 represented secondary spread. The patients with secondary cases included the two family members described above, a person who acquired it in his household from a Hong Kong visitor (representing 13% of cases), and a single healthcare worker (representing 4% of cases).

The single case in a healthcare worker was in a 32-year-old physician who cared for the wife of the initial case-patient. On March 14, the physician had performed a chest ultrasound that lasted approximately 30 minutes; he spent an additional hour in the room on March 17 during and

[1] On behalf of the SARS Advisory Committee.

after intubation. He was at the side of the bed supervising the intubation and in a direct line of droplet spread when the patient had episodes of coughing, sometimes partially sitting up. The physician reported wearing an N95 mask, eyeglasses without goggles, two pairs of gloves, and two gowns. His illness began on March 21, with clinical features that met the criteria for a probable SARS case and laboratory confirmation of coronavirus infection by RT-PCR. None of the other five persons present in the room for the intubation became ill after 28 days of follow-up.

SARS Control Measures in Taiwan

Beginning with the recognition of the first SARS case on March 14, Taiwan moved aggressively to isolate all suspected or probable case-patients in negative-pressure rooms and to equip all healthcare workers with enhanced protective equipment. Assistance from the U.S. Centers for Disease Control and Prevention (CDC) was requested, and a team has worked with Taiwan Center for Disease Control officials since March 16 to implement a framework for SARS control.

In March through April, 2003, Taiwan had a total of 764 negative-pressure rooms. Most were specially designed isolation rooms with HEPA-filtered air, negative pressure under continuous electronic monitoring, separate bathroom, and anteroom so that two doors separate the SARS patient from the rest of the hospital. The Center for Disease Control in Taiwan tracked available isolation rooms on an ongoing basis, and all of the first 23 probable case-patients were cared for in such negative-pressure rooms, located in 15 hospitals across the island. Resources in the highly affected areas of Hong Kong, Singapore, and Toronto may have been quite similar or superior to those in Taiwan. In contrast, neither of the two hospitals treating SARS patients in Hanoi had negative-pressure rooms, and such rooms were not commonly available in hospitals in Thailand, Laos, or other Asian countries (T. Uyeki, M. Simmerman, pers. comm.).

A team from the Taiwan Center for Disease Control visited each of the 15 hospitals caring for SARS patients to audit and implement strict infection control practices. Widespread education of healthcare workers on SARS control, with written guidelines, pictures, and demonstrations, was undertaken. Full protective equipment for healthcare workers was also widely available, and such equipment was provided and monitored under the conservative assumption, made early in the Taiwan outbreak, that airborne or fomite transmission of the agent should be presumed until proven otherwise. In addition to the handwashing and barrier precautions with gown, gloves, mask, and eye protection recommended by WHO, most healthcare workers in Taiwan used a disposable second layer of protective clothing (outer gloves, outer gown, head and foot covering) that were discarded before workers left the anteroom to prevent fomite transmission to other areas. Surgical or loose-fitting masks were actively discouraged, and N95 or greater filtration masks with tight-fitting seals were uniformly available. These precautions were used for 354 of 370 patient-care-days for the first 23 patients through April 14. After SARS was identified in the physician, use of goggles rather than glasses and careful fitting of masks were reemphasized to hospital infection control departments. Visits by family members were prohibited or minimized.

Active surveillance of exposed healthcare workers and contacts of patients was begun. In addition, the infection control nurses at the 15 hospitals passively monitored absenteeism in all employees. As of April 14, no other suspected or probable cases of SARS were identified through active daily monitoring of 525 healthcare workers, 210 work colleagues, 54 family and friends, and 31 public health staff who were exposed to the first 20 case-patients. Although some healthcare workers and household contacts of discharged patients had ongoing contact with SARS cases, as of this writing the outer limit of the incubation period (14 days) had already passed for contacts of 15 of the 23 cases, including 45 family and 220 work contacts. A more thorough epidemiologic investigation of close contacts was under way to determine the risk for transmission by level of contact and protection.

Beginning March 28, contacts of known patients, both suspected and probable, were quarantined at home for 10 days. As of April 14, a total of 1,572 persons had been put on home quarantine. This included healthcare workers exposed outside isolation settings, family and other close contacts, and those on airplanes with ill SARS patients if seated within three rows in front of or three rows behind a patient. Fourteen medical personnel were quarantined because they had had contact with two probable case-patients before they were hospitalized, including six present during endoscopy of one of the patients.

Conclusions

Approximately a 2-week interval elapsed between recognition of SARS outbreaks in Hanoi and Hong Kong and the introduction of SARS in Taiwan, giving clinicians and public health authorities in Taiwan some opportunity to act with more knowledge of its infectivity and severity. Available evidence suggests that the spread of SARS within Taiwan from the known imported cases was limited during the first 6 weeks after importation began. As occurred in some other SARS-affected areas, Taiwan initiated a strategy of aggressive public health measures combined with stringent hospital infection control practices that met, and in some instances exceeded, those recommended by WHO or CDC.

As of April 14, the SARS epidemic in Taiwan was at an early stage. Hospitals in Taiwan were able to care for all SARS patients in individual negative-pressure rooms; cohorting of patients in group wards was not necessary, and hospital staffing levels were not strained, as they have been in some cities with far more SARS patients. Also, although surveillance for healthcare worker transmission appeared thorough, other episodes of spread may have been missed. Concern about stigmatization and quarantine may have resulted in some concealment of illness not requiring hospitalization, and persons with mild illness or subclinical infection would not have been isolated. In part because of the challenge of ascertaining the true extent of transmission, health departments elsewhere have believed the SARS epidemics to be under control, only to find later that spread had occurred beyond what was recognized.

Taiwan continues to identify new cases in patients who report recent travel to mainland China and other SARS-endemic areas. Each year, more than 4 million visits are made by Taiwanese to mainland China for business and tourism (Bureau of Immigration, Ministry of the Interior, unpub. data), and the vast majority of all flights return through Hong Kong. Although such travel is being curtailed, Taiwan clearly will be at ongoing risk for importation and spread of SARS. Indeed, an apparent nosocomial cluster in Taipei was subsequently reported and remains under active investigation. The appearance of this cluster is sobering and underscores the fragility of SARS control measures in the setting of ongoing international spread of the disease.

Dr. Twu is currently Minister, Department of Health, Taiwan. His research has focused on the control of infectious diseases and nutritional deficiencies.

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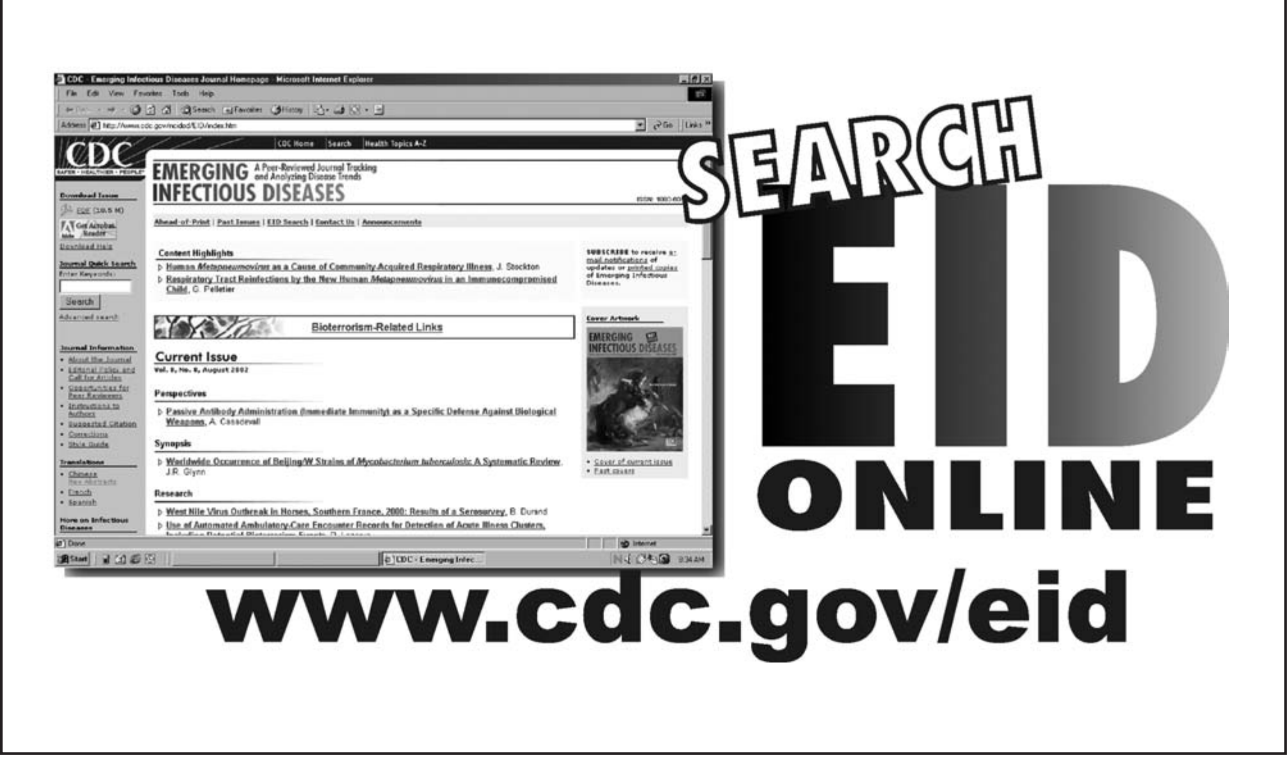

Human Rabies: A Reemerging Disease in Costa Rica?

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Two human rabies cases caused by a bat-associated virus variant were identified in September 2001 in Costa Rica, after a 31-year absence of the disease in humans. Both patients lived in a rural area where cattle had a high risk for bat bites, but neither person had a definitive history of being bitten by a rabid animal. Characterization of the rabies viruses from the patients showed that the reservoir was the hematophagous Vampire Bat, *Desmodus rotundus*, and that a sick cat was the vector.

Multiple studies in North and South America (1–3), Africa (4), Australia (5), Asia (6), and Europe (7) have documented the importance of rabies maintenance by various bat species. Aerosol transmission from bats was once considered as a possible mechanism in human rabies acquisition; however, because most patients lack a documented history of an animal bite, a more plausible explanation is that people, unaware of the risk of acquiring rabies from bat bites, are bitten by infected bats (8–10). The last human rabies case in Costa Rica, before the case we document, occurred in 1970, before the control of rabies in domestic animals had improved. The last documented case of canine rabies occurred in the North Pacific Coast (Salinas Bay) in 1987, and 26 cases of cattle rabies caused by Vampire Bat bites were reported during the 1990s. In September 2001, after 31 years without any known case of human rabies in Costa Rica, the National Children's Hospital reported a suspected case of paralytic rabies in a child referred from a regional hospital in the Brunca Region.

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Case Report

On September 12, 2001, a 9-year-old boy from La Gamba, Río Claro, in the Brunca Region of Costa Rica, was admitted to a local hospital with fever, cough, and malaise of 4 days' duration. On September 19, he returned to the hospital because of muscular weakness; he and was referred to the regional hospital on September 23 with acute flaccid paralysis that rapidly progressed to respiratory failure. The patient was intubated and transferred to the National Children´s Hospital with a diagnosis of viral encephalomyelitis. His severe central nervous system involvement progressed to coma in <24 h with muscle hypotonia. A computed tomographic scan showed generalized brain edema, and an electroencephalogram indicated diffuse and severe cerebral damage. The child died 6 days after hospital admission on September 29. A nuchal skin biopsy tested negative for rabies virus antigen by an immunofluorescence antibody assay, but postmortem brain tissue tested positive. Rabies virus was isolated by injecting mice with brain tissue from the child.

A field investigation was carried out to identify the likely mechanism of transmission. The house where the child had lived during the previous 4 months was in a wooded zone (Figure 1). In an interview, two members of the family noted that a 62-year-old woman who was in charge of the child died in a similar manner. On September 17, she exhibited malaise, muscular pain, headache, insomnia, and anxiety. On September 23, she was admitted to the local hospital, but because of the severity of her disease, she was transferred to the regional hospital on September 26. On September 29, she had sialorrhea, hemiplegia, and loss of muscular strength in her right arm; she also showed bizarre behavior and respiratory failure that progressed to apnea. She died on September 30. Characterization of the agents, conducted at the Centers for Disease Control and Prevention, demonstrated a rabies virus variant associated

Figure 1: Patient's home, located in an area where the risk of being bitten by a bat was high.

with vampire bats (*Desmodus rotundus*) from both patients, as described elsewhere in Latin America (11).

After the diagnosis of rabies was made, additional history was obtained about potential animal exposures. The family reported that during the middle of July 2001, their cat became very aggressive and demonstrated strange behavior. Their dog attacked the cat, and while the woman and child tried to stop the fight, the cat bit them. The dog killed the cat, and the carcass was discarded into a creek next to the house. (The body of the cat was not found during the investigation.) After the human cases were reported, the dog was confined with close observation, and in December 2001, it was euthanized. Postmortem brain tissue samples from the dog tested negative for rabies virus antigen.

Because rabies occurs in cattle in Costa Rica and because persons living in areas where contact with Vampire Bats is likely are at risk for of viral transmission, the Program of Animal Health of the Ministerio de Agricultura y Ganadería had implemented a Geographic Information System (GIS) during 1980. This animal surveillance system divided the country into 2,234 small areas (5 x 5 km²). Trained veterinary technicians used a national census of animals to register the number of weekly Vampire Bat bites from the farms included in each area. According to the frequency of Vampire Bat bites in cattle, the presence of rabies virus in populations of bats, and ecologic variables (e.g., temperature, precipitation, altitude, and land use), the GIS program defines areas of low, medium, and high-risk of disease (Figure 2). During August and September 2001, the GIS of animal health surveillance reported 169 cattle bites by Vampire Bats in the Brunca Region (rate 3.5%). Where the two patients lived, the rate of such attacks was 10.4%, and the area was classified as a high-risk zone for bat bites to cattle (Figure 3).

Rabies postexposure prophylaxis with a Vero cell rabies vaccine was administered to the relatives of the patients and to veterinary and healthcare personnel, who were in contact with the saliva of the patients. A retrospective search of other possible human cases was performed by analyzing the deaths with unknown origin in the discharge registry of the hospitals of the Brunca Region and the registry of the National Morgue. The case definition used was the following: any acute encephalopathy of unknown origin, with atypical focal neurologic signs or encephalomyelitis in any person who died between January 1 and September 30, 2001, in the Brunca Region. The search did not find any additional suspicious deaths associated with rabies in the region.

Conclusions

After 31 years without a case of human rabies in Costa Rica, the disease reemerged. Its reappearance reinforces the importance of an integrated network between veterinary and human health programs to identify risk zones of bat bites and to prevent human and animal cases. The active participation of combined health services in the early warning of the occurrence of suspected cases of emergent diseases is essential for effective, integrated surveillance. Human rabies must always be considered in the differential diagnosis of acute flaccid paralysis with consciousness impairment, even in patients without a known history of animal bite (giving priority to those in zones where cattle are at high risk for bat bites).

Rabies in bats was first reported during the 1920s. Since then, rabies has been confirmed in several species of bats. The historical association between Vampire Bats and livestock may tend to subvert the epidemiologic surveil-

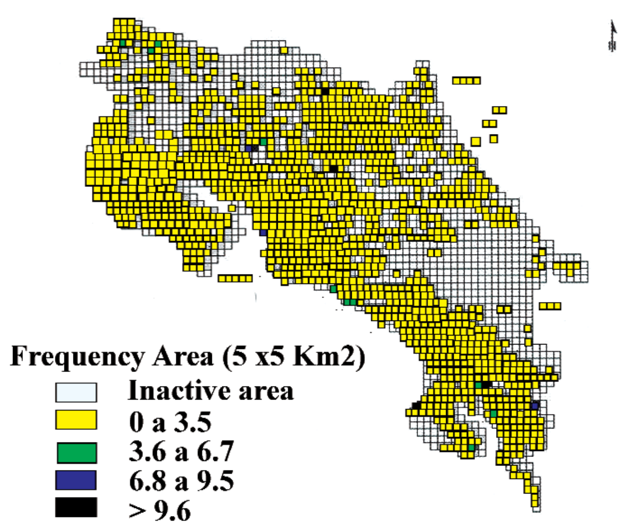
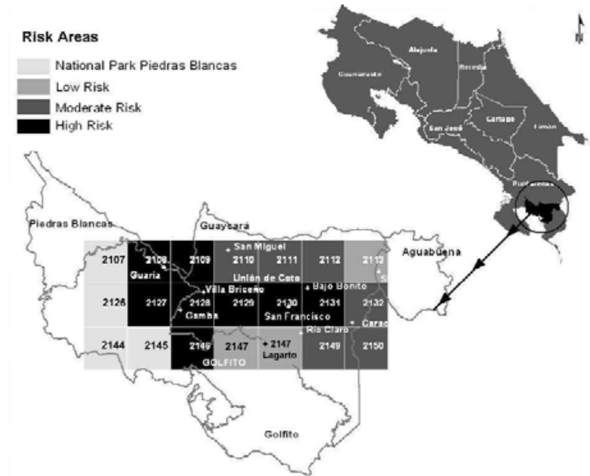


Figure 2. Annual prevalence of Vampire Bat bites, Costa Rica, 2001.



Source: Ministry of Agriculture, Animal Health Surveillance, Costa Rica

Figure 3. Human rabies cases, Costa Rica, September 2001.

lance in other species, such as domestic animals such as cats and dogs (12). This lack of association with other species could be due to a lack of intensified surveillance or to unknown factors related to the virus, host, or ecology.

Several studies have documented the transmission of rabies virus in the absence of a history of documented animal bite (13,14). No typical bite marks were observed in either of the patients, but the history of cat bite raises the possibility of an indirect manner of transmission, through the bite of another vector, previously infected by a bat. At the community level, the immigration of human populations throughout rural areas increases the risk of contact with hematophagous bats and facilitates the transmission of the virus to humans.

The presence of rabies in humans in Costa Rica associated with bat bites has demonstrated the importance of strengthening the integrated surveillance of animal and human health care. The GIS operation permits the identification of risk zones, so that measures for optimal control and prevention can be established. Participation of communities located in high-risk areas is a key strategy for the prevention and early detection of possible cases of rabies. Emergence of human rabies from infected wildlife, especially in countries where the disease has been eliminated in dogs, reinforces the need for maintaining awareness of both physicians and public health workers alike, as well as for improving laboratory tools needed for rapid rabies diagnosis.

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"The relation between art and science is closer than we might think, for despite the differences in approach, they are united by the imagination. In fact, the key to solving the theory of relativity came to Einstein as a visual image."

Janson and Janson, History of Art

Accidental Infection of Laboratory Worker with Vaccinia Virus

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We report the accidental needlestick inoculation of a laboratory worker with vaccinia virus. Although the patient had previously been vaccinated against smallpox, severe lesions appeared on the fingers. Western blot and polymerase chain reaction–restriction fragment length polymorphism were used to analyze the virus recovered from the lesions. The vaccinia virus–specific immunoglobulin G levels were measured by enzyme-linked immunosorbent assay. Our study supports the need for vaccination for laboratory workers that routinely handle orthopoxvirus.

The smallpox vaccine, formulated with vaccinia virus, is a highly effective immunizing agent. In 1980, the World Health Organization certified that the world was free of naturally occurring smallpox, and smallpox immunization programs were subsequently discontinued (1). Vaccination is still recommended for particular groups, namely, healthcare workers who handle materials potentially infected with vaccinia virus or other orthopoxviruses that infect humans (2).

The use of vaccinia virus in laboratories is likely to increase as a consequence of international concerns about the potential use of variola (smallpox) virus as a bioterrorism weapon. The vaccine is considered safe but can produce mild to moderate disease in vaccinees and can be disseminated to their close contacts (1,3,4). Accidental infections have also been reported. In 1991, an accidental infection with recombinant vaccinia virus was described after a needlestick injury on the left thumb of a laboratory worker (5). A case of vaccinia keratouveitis has been reported after accidental ocular autoinoculation from a recent vaccination site (6). We now report the accidental infection of a laboratory worker who manipulated vaccinia virus–infected cells.

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Case Report

A 26-year-old healthy laboratory worker, previously vaccinated against smallpox in childhood, sought treatment in March 2002 with a history of pain followed by the appearance of erythema and a pustule on the left thumb (Figure 1A). These symptoms appeared 3 days after she experienced an accidental needlestick while working with material from a vaccinia virus (strain WR)–infected cell culture during a virus purification procedure. Local symptoms worsened, and on the days 5 and 6, respectively, she noticed new pustules on the fourth and fifth fingers of the same hand (Figure 1E). Axillary lymphadenopathy was noticed on the day 6 after the accident. On day 8, necrotic areas around the lesions and a large erythemathous lesion appeared on the left forearm. On day 9 after inoculation, the local lesions worsened and amoxicillin/clavunate (1,750/250 mg per day) was administered because of a clinical suspicion of secondary bacterial infection (Figure 1B, F). The hand lesions were surgically excised to remove the necrotic tissue, and pustular fluid was collected for analysis (Figure 1C, G). After the surgical procedure, the patient improved slowly until she made a full recovery (Figure 1D, H), and the lesions healed in approximately 3 weeks.

Results

Pustular fluid from the lesions was collected and tested for the presence of bacteria and virus. The Gram stain and cultures were negative for bacteria. When a diluted sample of the pustular fluid was added to BSC-40 (monkey kidney) cell culture, a poxviruslike cytopathic effect was evident after 48 h of infection (data not shown). Vaccinia virus proteins were detected in infected cells by 12% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western Blot analysis with rabbit antiserum raised against total vaccinia virus proteins as described before (7). The protein profile was indistinguishable from that of the WR strain of vaccinia virus currently used in the laboratory (Figure 2A). The presence of vaccinia virus genome in the pustular fluid could be demonstrated by polymerase chain reaction (PCR), followed by restriction fragment length polymorphism (RFLP) of the phenol-chloroform–extracted DNA from BSC-40 cells infected with the clinical sample for 24 h at 37°C (8). Total DNA isolated from cells infected with the vaccinia virus–WR was used as reference. Two regions of the vaccinia virus genome were analyzed by using the following PCR primers: A24Rfwd 5′ATGAAAAAAACACT-GATTC and A24Rrev 5′TTACACCAGAAAAGACG-GCT; B9Rfwd 5′GACTAAATATTCATAA and B14Rrev 5′TACTAAAGTTCCGTCATC. The A24R gene was used as marker for the nonvariable region of the virus genome, and the PCR amplicons were digested with the endonucle-

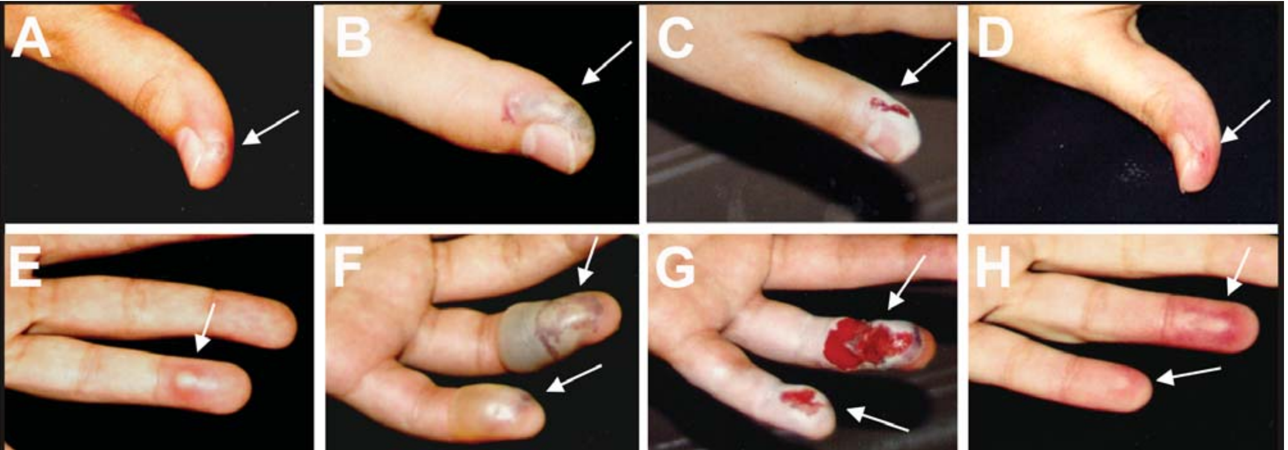

Figure 1. Progression of the local reaction on the left hand after accidental needlestick inoculation with vaccinia virus: thumb (A, day 4; B, day 11; C, day 12; D, day 20; fourth and fifth fingers (E, day 7, F, day 11; G, day 12; H, day 20). Lesions were surgically excised to remove necrotic tissue on day 11. Arrows indicate the lesion areas.

ases *Ssp*I and *Rsa*I (New England Biolabs, Beverly, MA, USA), as recommended by the manufacturer. The variable region of vaccinia virus genome was investigated by amplifying the DNA segment from the B9R to B14R genes and digestion of the amplicons with *Eco*R V and *Alu*I (Life Technologies, Rockville, MD, USA), as recommended. The digestion products were analyzed by using 1.2% agarose gels. The restriction patterns obtained for both regions in the test sample were identical to the profiles observed with the genome of vaccinia virus–WR (Figure 2B).

Serum collected from the patient day 20 after the initial inoculation was tested for vaccinia virus–specific immunoglobulin (Ig) G response by enzyme-linked immunosorbent assay (ELISA) as described (9,10). Purified vaccinia virus (1 µg/mL in 0.05 M carbonate buffer, pH 9.6) was used as the antigen, and the serum samples were diluted 1/100. Bound antibodies were detected with peroxidase-labeled, anti-human IgG (Biolab Diagnóstica, São Paulo, Brazil) dulated 1/8,000 as described (9,10). The optical density (OD) values were obtained with a microtiter plate spectrophotometer at 450 nm (BioRad, Model 3550 UV, Bio-Rad Laboratories, Hercules, CA, USA). The test serum specimen was compared to a panel of serum specimens from 22 unvaccinated persons and 11 persons who had been vaccinated some time previously, including a sample from the laboratory worker taken 6 years before the accident. When we compared the serum specimens collected before and after the accident, we observed an increase by a factor of 3.5 in the IgG-antibody response to vaccinia virus (Figure 2C). Furthermore, the vaccina virus–specific IgG levels in the test serum were 1.6 to 2.8 times higher than the levels in the panel of positive control samples and >5 times higher than levels in naive persons. Together, these results confirm that after the recent accident, a productive infection was found in the lesion and an immune response to vaccina virus was elicited.

Conclusions

Accidental infection with live pathogens by healthcare and laboratory workers has been frequently reported (11,12). The risk of infection cannot be avoided, although it can be prevented or minimized by safety measures. In some cases, vaccination of the workers is the best way to prevent the disease; however, vaccines are not always available.

We report the response of a laboratory worker to an accidental needlestick inoculation with vaccinia virus in 2002. After the accident, typical symptoms of vaccinia infection developed in the worker, followed by full recovery 4 weeks later. Vaccinia virus could be reisolated from the pustular fluid, and no major variation from the original seed virus was detected. Although the patient had been vaccinated against smallpox >20 years ago, a serum sample isolated 6 years before the accident showed a level of vaccina virus–specific IgG antibodies approximately 2 times higher than the level in naive persons. This level of humoral immunity was not able to prevent the progression of the infection as would be expected if she had been vaccinated recently. This result indicates that despite the high IgG levels induced after vaccina virus inoculation, persons vaccinated for >20 years are no longer fully protected against vaccina virus infection and could be vulnerable to variola virus or other orthopoxviruses that infect humans.

Nevertheless, we should consider some aspects of this accident that are not common in other situations (e.g., revaccination). The amount of virus in the needle before the accident was approximately 1,000 times higher than the amount in the vaccine preparations used for smallpox

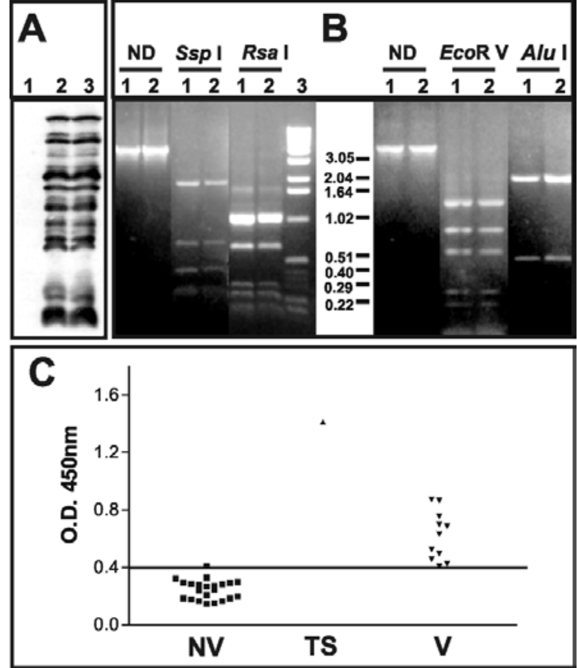

Figure 2. Characterization of the pustular fluid and serologic responses to vaccinia virus antigens. A) Western blot analysis of BSC-40 cells mock-infected (1); infected with vaccinia virus WR (2); or infected with 20 mL of the pustular fluid (3). Molecular weights are expressed in kDa. B) Polymerase chain reaction–restriction fragment length polymorphism analysis of vaccinia virus genome regions. Amplicons corresponding to the A24R gene or the segment between the B9R and B14R genes were digested or not (ND) with the restriction enzymes indicated on the top of the figure. (1) vaccinia virus–WR; (2) clinical sample. C) Detection of vaccinia virus–specific immunoglobulin G antibodies in serum samples from nonvaccinated (NV) and vaccinated persons (V) and the test subject (TS) was performed by enzume-linked immunosorbent assay, and the results are expressed as optical density 450-nm readings. The horizontal bar indicates the cut-off for the test.

vaccination (1). Even in a recently vaccinated person, a response to an infection of such high magnitude will most likely result in a local lesion. However, the question of whether a major reaction with severe symptoms would emerge in this hypothetical situation remains. Usually, a severe reaction has occurred only when a long period has elapsed after vaccination (1). Therefore, after a properly conducted risk assessment, laboratory workers vaccination should be considered as an occupational protection measure against accidental exposure to orthopoxviruses. The results of this study support the current Advisory Committee on Immunization Practices guidelines that recommend a 1-year vaccination regimen for workers who handle low-virulence poxvirus and a 3-year regimen for workers that handle high-virulence strains.

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Anthroponotic Cutaneous Leishmaniasis, Kabul, Afghanistan

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A prevalence survey in Kabul City showed that 2.7% and 21.9% of persons have active leishmaniasis lesions or scars, respectively. Incidence of disease was estimated to be 2.9% (29 cases/1,000 persons per year; 95% confidence interval 0.018 to 0.031). Disease was associated with age and gender; logistic regression analyses showed significant clustering of cases.

In Afghanistan, the majority of leishmaniasis cases are caused by *Leishmania tropica* (1), which is transmitted anthroponotically (i.e., humans are the reservoir) by the sandfly (*Phlebotomus sergenti)* (2). Anthroponotic cutaneous leishmaniasis (ACL) is generally characterized by large and/or multiple cutaneous lesions with a variable tendency to self-cure (3). Because of sandfly exposure, most lesions occur on the face, often leading to severe stigmatization in affected persons (e.g., women with lesions are often deemed unsuitable for marriage or raising children). Anecdotal reports suggest that because of the political instability and destruction of the housing and health infrastructure during the Mojahedin and Taliban regimes, *L. tropica* cases have increased during the past decade, with current World Health Organization (WHO) estimates of 200,000 ACL cases in Kabul alone (4). Also, because of several factors (the mass migration of *L. tropica*–infected [and infectious] Afghan refugees; the sporadic treatment of ACL cases by WHO and nongovernmental organizations; and the virtual absence of vector-control strategies), *L. tropica* has spread to areas where ACL was previously nonendemic (e.g., northwestern Pakistan) (5).

The Study

Before developing an ACL-control strategy, we conducted a cluster-based, house-to-house survey in Kabul City between July and September 2001 to collect data on the extent of ACL. The leishmaniasis transmission season is between April and October. Each of the city's 14 districts was divided into random clusters according to the district's population size, for a total of 90 sample clusters; 30 neighboring households were surveyed in each cluster, with the first household selected at random. A team of medical staff diagnosed disease in household members on the basis of presence or absence of ACL lesions or scars, number of lesions, and date of lesion onset; members were interviewed to collect demographic data such as gender and age. Because of logistic constraints, parasitologic diagnosis of ACL lesions (e.g., microscopic examination or parasite culture) was not carried out. However, in Afghanistan, ACL-like skin lesions from other causes are rare, and our experience suggests that clinical diagnosis has a sensitivity and specificity of >80% and >90%, respectively (Reithinger et al., unpub. data). Written approval to conduct the study was obtained from the Afghan Ministry of Public Health. Informed consent was obtained from study participants; all persons with active cases surveyed were offered free antileishmanial treatment at the HealthNet International clinic.

Of 26,892 persons surveyed, 726 (2.7%) and 5,900 (21.9%) had active leishmaniasis lesions or scars, respectively. Of those persons with ACL lesions, the mean lesion number was 2.4 (range 1–50) and the mean lesion duration (to survey date) was 9.1 months (range 0.1–96). A total of 26,887 observations, with full disease and demographic records, from 2,683 households from the 90 sample clusters, were used in logistic regression analyses with a binary outcome variable (ACL lesion or scar).

Four variables were created to assess the distribution of leishmaniasis cases: the prevalence of active lesions in other members of the same household, the prevalence of scars in other members of the same household, the prevalence of active lesions in the nearest neighbor households, and the prevalence of scars in the nearest neighbor households. The nearest neighbor households were defined in terms of the survey protocol. In a given sample cluster, the nearest neighbor to household 1 was household 2; the nearest neighbors to household 2 were households 1 and 3; and so forth. The logistic regressions with robust standard errors (i.e., clustering of households to control for within-household correlations) were also adjusted for age (continuous to year of age at last birthday), gender, and sample cluster (categorical 90 levels). All analyses were conducted in STATA 7 (Stata Corporation, College Station, TX).

The regression analyses show that female patients are at significantly higher odds of having leishmaniasis lesions or scars (Table). Odds of disease were associated with age: elderly people are at slightly greater risk of having active lesions; and elderly persons are less likely to have leishmaniasis scars (the drop in scar prevalence in persons ≥12 years was significant, odds ratio: 0.994 (95% confidence interval [CI] 0.991 to 0.997), p<0.001). The analyses showed significant clustering of ACL within and between

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Table. Within- and between-household clustering of anthroponotic cutaneous leishmaniasis lesions and scars, Kabul City, Afghanistan[a]

Explanatory variables	Outcome variable[b]	
	Lesion	Scar
Within-household prevalence	OR (CI)	OR (CI)
Prevalence of lesions in other household members	132.3 (67.3 to 259.8) p<0.001	3.728 (2.799 to 4.964) p<0.001
Prevalence of scars in other household members	1.988 (1.500 to 2.635) p<0.001	48.24 (41.79 to 55.68) p<0.001
Between-household prevalence		
Prevalence of lesions in nearest neighbor households	2.323 (0.984 to 5.486) NS	1.585 (1.036 to 1.353) p<0.05
Prevalence of scars in nearest neighbor households	1.376 (0.957 to 1.980) NS	1.184 (1.036 to 1.353) p<0.05
Other		
Age	1.005 (1.000 to 1.009) p<0.05	1.013 (1.011 to 1.015) p<0.001
Sex (female relative to male)	1.383 (1.177 to 1.626) p<0.001	1.186 (1.108 to 1.270) p<0.001
Sampling area[c]	Chi square=213.6, d.f.=89 p<0.001	Chi square=330.8, d.f.=89 p<0.001
Overall model fit	Pseudo R^2 = 11.89% p<0.0001	Pseudo R^2=22.72 p<0.0001

[a]Abbreviations used: OR, odds ratio; CI, 95% confidence intervals; d.f., degrees of freedom; NS, not significant.
[b]The statistical model controlled for age (in years), sex, and sampling area (a total of 90 areas), while the standard errors were adjusted for sampling of persons at the household level.
[c]The overall significance of the variable is shown rather than the ORs for the 90 different sampling areas.

households (Table). A person's probability of having an active lesion was increased greatly if other lesions appeared on persons in the same household (Figure, A) and also (but less so) if other scars occurred on persons in the same household. A person's probability of having a scar was greatly increased if persons with active lesions or scars were present in the same household (Figure, B). Finally, a person's probability of having a scar was greatly increased with the presence of persons with active lesions or scars in neighboring households (Figure, C). No signif-

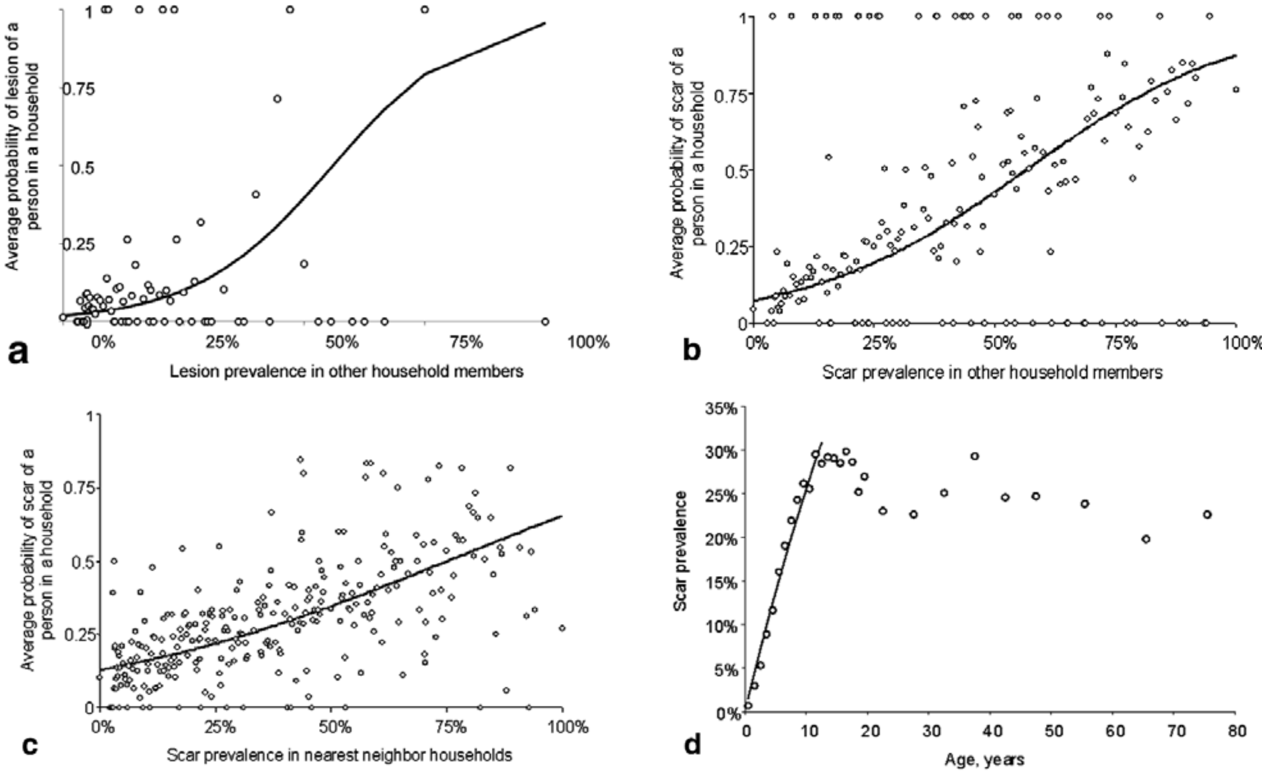

Figure. A, the average probability of having a lesion at different levels of lesion prevalence recorded among other members of the same household (open circles) and the unadjusted fit (solid line) from the logistic regression. B, the average probability of having a scar at different levels of scar prevalence recorded in other members of the same household (open circles) and the unadjusted fit (solid line) from the logistic regression. C, average probability of having a scar at different levels of scar prevalence in nearest neighbor households (open circles) and the unadjusted fit (solid line) from the logistic regression. D, force of infection, λ, can be estimated from the age-prevalence data, where the proportion, P, of persons with ACL at age *a* (where *a* is age at last birthday plus 0.5 years) is given by $P(a) = 1-\exp(-\lambda a)$ (6). If one assumes that age-independent transmission started 12 years earlier (1), λ was estimated by maximum likelihood by using the observed age-prevalence data for children $\leq$12 y of age.

icant clustering of ACL lesions occurred between households; however, the sample size was small for analyses. Overall, these findings are consistent with clustering of ACL transmission, including transmission in areas where previous transmission has occurred (because of association with scar prevalence). When maximum likelihood methods (6) are used, the average annual force of ACL infection (λ) was estimated to be 0.029 per year (29 cases/1,000 persons per year; 95% CI 0.028 to 0.031) over the past 12 years (Figure, D).

Conclusions

Currently six clinics provide leishmaniasis diagnostic and treatment services in Kabul; an estimated 20% of the total 67,500 patients (based on the observed prevalence of 2.7% and a total 2.5 million population for Kabul) are diagnosed and treated. Whether this fact alone could explain the extent and duration of the leishmaniasis epidemic in Kabul is uncertain. Our analyses show that persons are at high risk for active ACL when a high proportion of persons with ACL scars are in the same or neighboring households. The likely explanation for this finding is that sandfly distribution and abundance are patchy but stable over time. The high prevalence of persons with active ACL in Kabul and the comparatively high ACL incidence show that ACL-control strategies (e.g., increasing the number of clinics providing treatment facilities or providing personal protection methods against sandflies) should be conducted soon. We demonstrate that a blanket-coverage ACL-control strategy is not necessary: transmission of this disease is focalized, and interventions (e.g., household insecticide spraying, insecticide-impregnated bednets or chaddars) (5) targeting households with a high proportion of persons with leishmaniasis lesions or scars or city districts containing a high number of high transmission clusters should have a major impact on transmission in Kabul.

The international donor community often considers ACL to be of peripheral importance (e.g., the disease was not included in the basic package of health services for Afghanistan) (7) because this disease has no impact on death rates and patient treatment costs (usually U.S.$15–200 [8]) are not recovered. Failure to implement a control strategy for this disease will likely lead to an increase in its impact and social stigmatization and represent further problems for a health infrastructure already crippled by 20 years of war.

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Fluoroquinolone Use and *Clostridium difficile*–associated Diarrhea

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We performed a case-control study to evaluate the association between antibiotic use and *Clostridium difficile*–associated diarrhea (CDAD), matching for admission unit and time at risk for CDAD. A multivariable regression model showed that treatment with fluoroquinolones (odds ratio 12.7; 95% confidence interval 2.6 to 61.6) was the strongest risk factor for CDAD.

Clostridium difficile–associated diarrhea (CDAD) is a leading cause of nosocomial diarrhea in the United States (1–4). On average, compared with patients without this disease, patients in whom CDAD is diagnosed have hospital stays that are 3.6 days longer and additional hospital costs of $3,699 (5). Research has shown that patients are usually exposed to *C. difficile* throughout their hospitalizations and that antibiotic use promotes the acquisition of this organism (1). The outcome of acquisition, which may be colonization or infection with *C. difficile,* is thought to be determined primarily by patient factors including advanced age and severity of underlying illness, which may compromise the ability to mount an immune response against the bacteria (6).

Clindamycin, penicillins, and cephalosporins have been associated with CDAD (4). Although fluoroquinolones are not currently believed to cause this illness, several case reports of fluoroquinolone-associated *C. difficile* diarrhea have been published (6–12). A case-control study of patients at an acute-care hospital identified ciprofloxacin use as a strong risk factor for nosocomial CDAD (13). The broadened anti-anaerobic spectrum of newer fluoroquinolones raises the issue of whether therapy with these agents can predispose this illness to develop in patients (14).

Increasing rates of *C. difficile* infection in cases dispersed throughout our healthcare system prompted an examination of patient-associated risk factors for CDAD. We hypothesized that patients in whom CDAD was diagnosed were more likely to have received antibiotics of which use had increased over the past year and that differences in antimicrobial drug–prescribing patterns could account for the observed increase in cases.

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The Study

The Veterans Affairs Maryland Health Care System (VAMHCS) provides all medical services from intensive care to ambulatory and pharmacy services for approximately 36,000 veterans at four separate inpatient sites. A total of 778 beds are available for inpatient care, 120 of which are dedicated to acute medical and surgical care. Cases were defined as patients who were admitted to a VAMHCS institution from January 1, 2001, to June 30, 2001, who had a new onset of diarrhea documented in their medical records at least 72 hours after admission, a subsequent positive *C. difficile* toxin A enzyme immunoassay result (Wampole Laboratories, Cranbury, NJ), and no known history of CDAD. Patients with other reasons for diarrhea, such as laxative use, were excluded. The date of the positive *C. difficile* toxin test was considered to be the date of CDAD diagnosis.

We selected two controls per case from patients admitted to a VAMHCS institution for at least 48 hours during the same 6-month period as the case-patients. Controls were matched to the case-patients by unit of admission and length of time at risk for development of CDAD (defined below). We attempted to find two controls for each case with a time at risk within 5 days of that of the case. When finding such a control was not possible, we selected a control with the next closest length of time at risk. Controls had no known history of CDAD and did not receive oral metronidazole during their hospital stay in order to minimize misclassification of controls that might be cases.

We collected data by reviewing electronic medical records. Since antibiotic use up to 8 weeks before the CDAD diagnosis has been implicated as causing infection in previous studies (1,3), we examined both inpatient and outpatient antibiotic use within the 6 weeks before diagnosis of CDAD for cases and for 6 weeks before hospital discharge for controls. Specific use of clindamycin, cephalosporins, fluoroquinolones, piperacillin-tazobactam, and any other antibiotic drugs was recorded. The number of days that fluoroquinolones were administered to each patient was determined from medication orders and nursing notes. Length of time at risk for CDAD was defined as the number of days from admission to development of the illness for cases and the number of days from admission to discharge for controls. Demographic variables and details of hospital admission were also recorded, including the unit where CDAD was diagnosed (cases) or the admission unit (controls).

We compared characteristics of cases and controls with the Wilcoxon rank-sum test for continuous variables and

the Fisher exact test for categorical variables. Matched analysis of the association between individual variables and case or control status was performed by using Cochran-Mantel-Haenszel estimates. Conditional logistic regression was used to assess the odds of CDAD developing in a patient. Variables significantly associated with CDAD in our preliminary analysis were included in the multivariable regression model. Confounding was assessed by checking for a ≥10% change in the coefficient estimate of covariates between models. A p value ≤0.05 was considered significant; all statistical tests were two-tailed. Statistical analyses were performed by using SAS software version 8.1 (SAS Institute, Inc., Cary, NC).

Thirty patients met the case definition during the study period; 60 controls were selected. The mean age of cases and controls was not significantly different (Table 1). With the exception of one female control, all patients were male. Despite matching, case-patients had a longer length of time at risk for CDAD, but the difference between cases and controls was not statistically significant (p=0.18). Of both cases and controls, 20% were admitted to general medical units, 23% to general surgical units, 27% to subacute or long-term care, 17 % to the medical intensive-care unit, and 13% to the surgical intensive-care unit.

All 30 (100%) case-patients received antibiotics during the 6 weeks before their CDAD diagnosis. In the comparable 6-week window, 38 (63%) of the controls received antibiotics (p<0.01 for difference). Both clindamycin and fluoroquinolones were administered to a significantly higher proportion of cases than controls. For the patients who received fluoroquinolones, levofloxacin was prescribed most often for both cases (60%) and controls (60%), followed by ciprofloxacin (45% and 27%, respectively), and gatifloxacin (14% and 20%, respectively). These differences were not statistically significant. Among the patients who received fluoroquinolones, 41% of case-patients and 27% of controls received >1 week of fluoroquinolones (p=0.01).

Matched univariate analysis of risk factors for CDAD showed that fluoroquinolone use (odds ratio [OR] 13.5; 95% confidence interval [CI] 3.1 to 58.8) and clindamycin use (OR 3.1; 95% CI 1.0 to 9.4) were associated with developing this illness (Table 2). The results of the multivariable analysis are shown in Table 3. After confounding from other antimicrobial agents was controlled for, fluoroquinolone use was significantly associated with an increased risk of developing CDAD (OR 12.7; 95% CI 2.6 to 61.6).

Conclusions

Although ciprofloxacin-induced CDAD has been reported, early reports were dismissed as being due to other causes of diarrhea, including infection with *Salmonella* and previous treatment with a different antibiotic (6–8,12). A group of bone marrow transplant patients who received ciprofloxacin monotherapy for prophylaxis against infection had no instances of CDAD, but two concurrent reports included cases of CDAD associated exclusively with ciprofloxacin (9,10,15). Another report implicated levofloxacin in eight of nine cases of CDAD in a nursing home (11). Fluoroquinolone use was also identified as an independent predictor of a positive *C. difficile* toxin assay in hospitalized patients (16). In addition, a case-control study of patients at an acute-care hospital identified ciprofloxacin use as a strong risk factor for nosocomial CDAD with an OR >5 in each regression model

Table 1. Characteristics of CDAD cases and matched controls, Veterans Affairs Maryland Health Care System, January 1, 2001–June 30, 2001[a]

Characteristic	Cases (n=30)[b]	Controls (n=60)[b]	p value
Age, median y	72 (66–79)[c]	67 (56–76)[c]	0.30
Diagnosis causing admission			
Infectious	11 (37)	11 (18)	0.07
Cardiovascular	5 (17)	14 (23)	0.59
Neurologic/psychiatric	3 (10)	15 (25)	0.16
Gastroenterologic	2 (7)	4 (7)	1.00
Respiratory	1 (3)	5 (8)	0.66
Other	8 (27)	11 (18)	0.42
Antibiotics within 6 weeks	30 (100)	38 (63)	<0.01
Cephalosporins	7 (23)	20 (33)	0.30
Clindamycin	9 (30)	7 (12)	0.03
Fluoroquinolones	22 (73)	15 (25)	<0.01
Piperacillin/tazobactam	12 (40)	18 (30)	0.30
All other antibiotics	17 (57)	27 (45)	0.30
Days at risk for CDAD, median	21(10–30)[c]	13(7–25)[c]	0.18

[a]CDAD, *Clostridium difficile*–associated diarrhea.
[b]Unless otherwise indicated, values in parentheses show percentages.
[c]Value in parentheses shows interquartile range.

Table 2. Matched univariate analysis of risk factors for *Clostridium difficile*–associated diarrhea

Risk factor	Odds ratio	95% confidence interval	p value
Fluoroquinolone	13.5	3.1 to 58.8	<0.01
Clindamycin	3.1	1.0 to 9.4	0.05
Piperacillin/tazobactam	1.9	0.7 to 5.1	0.24
Cephalosporins	0.6	0.2 to 1.7	0.32
All other antibiotic drugs	1.6	0.7 to 4.1	0.28

(13). Thus, our study is consistent with more recent reports that implicate fluoroquinolone use as a risk factor for CDAD.

We found that the association between fluoroquinolones and CDAD is stronger than the association between clindamycin and CDAD. However, the confidence intervals are wide because of the small sample size and overlap for the estimates, making a conclusion that fluoroquinolones are a stronger risk factor for CDAD than clindamycin inappropriate from our study. Because patients commonly receive more than one antibiotic, accurately measuring the effects of individual antibiotics in an observational study is difficult. Concurrent prescribing of clindamycin and fluoroquinolones may have biased the estimates of the OR; however, only 32% of patients who received fluoroquinolones also received clindamycin. In the case-control study of acute-care patients by Yip et al., ciprofloxacin was also a stronger risk factor than clindamycin (13).

Our study has a number of limitations. Since the study was retrospective and we did not perform surveillance cultures for *C. difficile,* we could not ascertain when this organism was acquired; however, all cases received antibiotics before the diagnosis of CDAD. Thus, we conclude that fluoroquinolones are clearly associated with *C. difficile* infection. On the basis of our study design, we could not determine whether fluoroquinolones increase acquisition or promote infection once *C. difficile* is acquired. Although we did not specifically assess the role of patient-to-patient transmission in this study, we selected case-patients and controls from the same hospital units and with a similar risk period for developing the illness. Given the strength of the association between fluoroquinolone use and CDAD, more precise controlling for patient-to-patient transmission is unlikely to eliminate the association.

If fluoroquinolone use is a stronger contributing factor to *C. difficile* infection than other antibiotics, then restriction of fluoroquinolone use among inpatients would result in decreased CDAD rates. Climo et al. reported a decrease in the incidence of CDAD at their institution after implementing a formulary restriction of clindamycin (17). However, the decision to restrict fluoroquinolone use would need to be weighed against the clinical advantages of using fluoroquinolones, such as convenient dosing and excellent oral bioavailability (i.e., the ability of a drug to achieve high serum levels when taken by mouth). We observed a strong association between fluoroquinolone use and CDAD in both our acute-care and long-term–care patients, which supports a number of reports implicating fluoroquinolones in the development of CDAD (6–11,13). A prospective study of this association is warranted, given the increasing use of fluoroquinolones and the excess complications and costs associated with *C. difficile* infection (5).

Table 3. Multivariable model of risk factors for *Clostridium difficile*–associated diarrhea in cases (n=30) versus controls (n=60), controlling for days at risk

Risk factor	Odds ratio	95% confidence interval
Fluoroquinolones	12.7	2.6 to 61.6
Cephalosporins	0.4	0.1 to 1.5
Clindamycin	2.2	0.5 to 9.1

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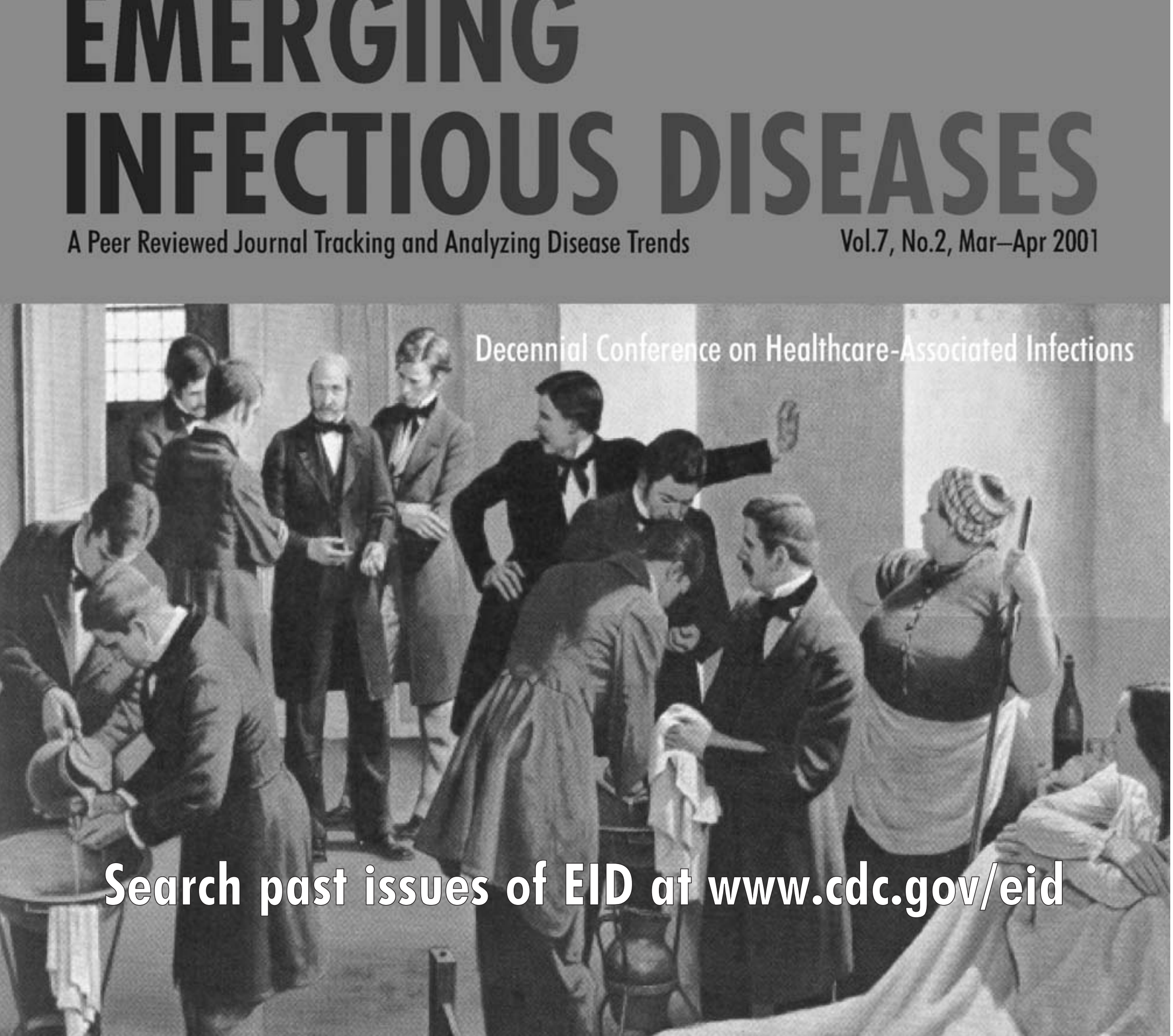

Absence of *Neisseria meningitidis* W-135 Electrophoretic Type 37 during the Hajj, 2002

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We document the absence of carriage of *Neisseria meningitidis* W-135 of the sequence type 11 in returning pilgrims after the Hajj 2002. This finding contrasts with the 15% carriage rate we previously reported in pilgrims returning from the Hajj 2001. The epidemiology of carriage may be changing or may have been controlled by vaccination and a policy of administering antibiotics to pilgrims from countries with a high incidence of meningococcal disease.

Two million pilgrims from all over the world congregate for the annual Islamic pilgrimage to Mecca and Medina in Saudi Arabia (Hajj pilgrimage). Because overcrowding facilitates person-to-person transmission of meningococci, this pilgrimage has been associated with meningococcal disease outbreaks (1–3). An international outbreak of serogroup W-135 meningococcal disease occurred during the Hajj pilgrimages in 2000 and 2001 (4,5). The outbreak-associated W-135 strains were of a single clone of the electrophoretic type (ET)-37 complex (6) and were closely related to other meningococci with an established propensity to cause disease clusters (7). Several reports from all over the world have shown that this W-135 outbreak strain affected not only pilgrims but also household contacts of returning pilgrims and the community at large, with the potential of non–Hajj-related further epidemics (8–12). As nasopharyngeal carriage is the primary source of transmission (13), worldwide dissemination is thought to be due to pilgrims who acquired W-135 carriage during the pilgrimage and introduced the strain on return to their countries of origin (14). We previously reported a 15% carriage rate of *Neisseria meningitidis* W-135 in Singaporean pilgrims returning from the Hajj 2001, despite the fact that all were vaccinated with quadrivalent polysaccharide meningococcal vaccine (15). W-135 carriage was substantially transmitted to unvaccinated household contacts (15,16). Pulsed-field gel electrophoresis (PFGE) of these isolates showed that they were distinguishable in 83% of the samples (unpub. data), indicating that this outbreak had a clonal origin. The high transmission rate of W-135 carriage from pilgrims to household contacts translated into a high attack rate of W-135 meningococcal disease among contacts in 2001 (17). We investigated carriage rates in pilgrims returning from the 2002 Hajj to determine whether W-135 was still a problem, and we compared the strains from 2002 with those from 2001 to document the evolving molecular epidemiology of Hajj-associated W-135 strains.

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The Study

We conducted a prospective study on the acquisition of meningococcal carriage in Singaporean Hajj pilgrims on the Hajj 2002. We used the same design and methods as we used in our previous study for the Hajj 2001, and the study was performed by the same research and laboratory staff (15). Pilgrims were recruited consecutively at the time of vaccination with quadrivalent meningococcal and influenza vaccine at a Muslim center that performs mass vaccinations for pilgrims referred by numerous national Muslim travel agencies. A swab sample was taken from both tonsils and the pharyngeal wall, by using a standard technique, and immediately transferred to a plate of selective culture medium (Oxoid GC, Basingstoke, UK). Repeat swab samples were taken 2 weeks after return from the pilgrimage. Returning pilgrims were asked if the symptoms of upper respiratory tract infection had occurred and if antibiotic drugs were taken during the pilgrimage, specifically a single dose of ciprofloxacin. All study participants gave written informed consent. The study was approved by the Ethics Committee of Tan Tock Seng Hospital.

Culture plates were immediately put in candle jars, transferred to the laboratory within 2 to 4 hours of collection, incubated at 37°C in humidified air with 5% carbon dioxide, and examined for bacterial growth at 24 and 48 hours (18). Identification of isolates as *N. meningitidis* was performed by traditional methods and was confirmed by API NH (BioMerieux SA, Lyon, France). The serogroup was determined by slide agglutination with polyvalent sera and serogroup-specific sera (A, B, C, D, Y, W-135, X, and Z) (Murex, Dartford, UK).

PFGE was performed on all meningococcal isolates by using previously described methods (19). The restriction enzyme used was *Spe*I. Multilocus sequence typing (MLST) was performed on all W-135 isolates, as well as on stored isolates from the 2001 Hajj, as described by Maiden et al. (20) by sequencing of seven housekeeping genes. Primers, determination of sequence alleles, and designation of sequence types are described on the MLST Web site (available from: URL: http://neisseria.org/

nm/typing/mlst). Participant demographics, duration of stay at the Hajj, respiratory symptoms, antibiotic intake, carriage rates, and results of PFGE and MLST were compared with the findings by our group from Singaporean pilgrims returning from the Hajj 2001 (15).

Conclusions

Tonsillopharyngeal swab samples were taken from 193 Malay pilgrims at a median time of 30 days (range 18–52) before their departure for the Hajj. One hundred fifty-three (79%) had a repeat swab sample taken at a median time of 10 days (range 2–17) after their return from the Hajj. The mean age was 48 (SD 8.07) years, and 48% were male. The mean duration of stay was 33 days (range 14–41, SD 3.6). Returning pilgrims reported cough (70%) and use of antibiotics (52.9%). Nine percent of pilgrims reported ciprofloxacin use.

Four of the pilgrims (2.6%) were carriers before the Hajj. Three isolates could not be grouped; one was serogroup B. Two (1.3%) of the returning pilgrims were carriers, and both isolates were serogroup W-135. The PFGE patterns of the two isolates of W-135 differed from each other by >10 bands, and each differed by >7 bands from the predominant PFGE pattern identified in the 2001 Hajj (15).

On MLST, these two isolates were sequence type (ST)-192 and ST-32 and shared only one of seven alleles with each other. They shared none of the seven alleles with the isolates from the 2001 Hajj. Thirty (94%) of the 32 isolates from the 2001 Hajj pilgrims were ST-11. The Table summarizes the results from the 2002 versus 2001 Hajj pilgrimage.

We documented a low W-135 meningococcal carriage rate (1.3%) in pilgrims returning from the 2002 Hajj, which is in stark contrast to the 15% carriage rate in pilgrims returning from the year 2001 Hajj (15). On multilocus sequence typing, these isolates were ST-192 and ST-32 and not ST-11, which was the dominant sequence type in returning pilgrims from the year 2001 Hajj. ST-11 is most commonly associated with the hypervirulent ET-37 complex (6,7). Our strains from the year 2001 Hajj are therefore highly likely to be the same as the ET 37 ST-11 responsible for the outbreak in Saudi Arabia (6,7,9). The two W-135 strains (ST-192 and ST-32) isolated in pilgrims returning from the 2002 Hajj were clearly distinct, both in PFGE and MLST, from the W-135 strain (ST-11) isolated in pilgrims returning from the year 2001 Hajj (15) and are thus unlikely to have evolved from ST-11 strains circulating in 2001. Genetically distinct Hajj-compatible phenotypes were also reported in France in 2002 (21).

While carriage rates are indicative of the potential of a meningococcal outbreak, occurrence of disease is ultimately more informative (21). The absence of the hypervirulent W-135 ET ST-11 in returning Singaporean pilgrims from the Hajj 2002 is reflected by an absence of Hajj-associated clinical cases of W-135 disease in Singapore in 2002. This finding is also consistent with international reports that showed a marked decrease in cases of meningococcal disease in 2002 caused by the Hajj 2000/2001 outbreak strain (21). Therefore, in 2002, the W-135 carriage was probably also low in pilgrim populations other than these from Singapore.

The marked difference in W-135 carriage rates is unlikely to be due to selection bias or study methods, as we used the same recruitment strategy, swabbing techniques, and laboratory methods in 2002 and 2001. Although the decrease in carriage in the year 2002 could reflect spontaneous changes in epidemiologic features of the outbreak, a number of public health interventions may have played a role. Influenza vaccination was given in the 2002 cohort but not in the 2001 cohort, and respiratory symptoms are known to promote meningococcal transmission (22). However, these interventions are unlikely to account for the difference in carriage: frequency of upper respiratory symptoms were similar (or even more frequent) in the pilgrims of 2002 compared to the pilgrims of 2001. More rigorously enforcing a long-standing policy to administer antibiotics to pilgrims from Africa upon entry to Saudi Arabia (5), plus the recent extension of this policy to

Table. Characteristics of pilgrims returning from the Hajj 2002 compared with those returning from the Hajj 2001[a]

Characteristics	Returning Hajj pilgrims 2002 (n=153)	Returning Hajj pilgrims 2001 (n=171) (ref. 15)	p value
Median age (yrs)	48	48	
Gender (male) (%)	48	46	
Race (Malay) (%)	98	98	
Median interval between return from pilgrimage and throat swab in days (range)	10 (2–17)	17 (1–45)	
Median duration of pilgrimage in days (range)	33 (14–41)	33 (3–47)	
Overall meningococcal carriage rate (%)	1.3	17	<0.001
Carriage of W-135 ST 11 (%)	0	15	<0.001
Cough (%)	75	56	NS
Antibiotic drug use (%)	56	41	NS

[a]NS, not significant.

incoming pilgrims from the Indian subcontinent (23), may have played a role in decreasing the importation of W-135 to the pilgrim pool. In addition, more liberal use of ciprofloxacin may have occurred during the 2002 Hajj. Nine percent of the pilgrims in our cohort reported ciprofloxacin use.

However, the main difference between the year 2002 and 2001 Hajj pilgrimages was that coverage with quadrivalent meningococcal polysaccharide vaccine was only partial in 2001 (9), whereas almost complete vaccine coverage for this pilgrimage can be assumed for the year 2002 after this vaccine became a Hajj visa requirement for all pilgrims (21). Although polysaccharide vaccine does not prevent acquisition of carriage (13), as confirmed in our 2001 cohort (15), polysaccharide vaccines can induce transient reduction of carriage (24). In addition, vaccines reduce the incidence of meningococcal disease and thus circulation of meningococci. Almost complete vaccine coverage of 2 million pilgrims may have therefore contributed to a decrease in the spread of carriage within this pilgrim population and consequently reduced transmission of carriage from returning pilgrims to their contacts.

The significant decrease of W-135 meningococcal carriage and the absence of ST-11 in returning pilgrims from the Hajj 2002 are important findings with regard to public health policy. Our documented low W-135 carriage rate in Singaporean pilgrims in the year 2002 is indicative of a decreased potential for spread of meningococcal disease in close contacts of returning pilgrims. On the basis of the high carriage rate in returning pilgrims, together with the transmission to household contacts and observed secondary cases in the community in 2001 (16,17), we suggested that administrating antibiotics to returning pilgrims would be appropriate (15). Indeed, the Saudi Arabia authorities have implemented such a policy for their returning Saudi pilgrims (23). However, the epidemiologic features of carriage may be changing or has been controlled by vaccination and administering antibiotic drugs to incoming pilgrims from countries with high incidence. Thus, the administration of antibiotics to all returning pilgrims appears to be unnecessary at the present time. Ongoing surveillance of carriage rates both in the resident population in Saudi Arabia as well as in arriving and departing pilgrims is paramount for rapid readjustment of a policy to administer antibiotics to eradicate carriage.

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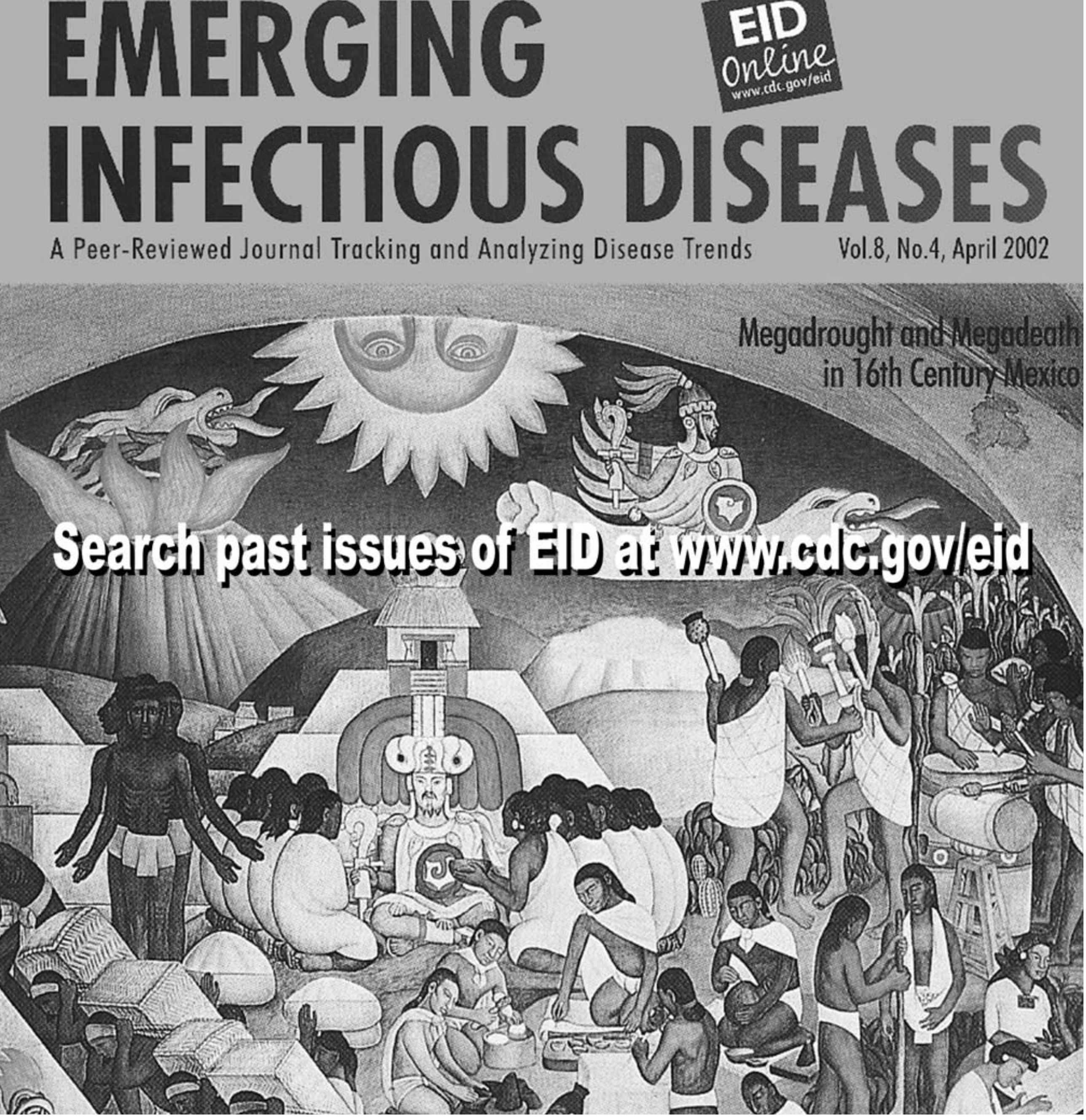

Laboratory Surveillance of Dengue in Argentina, 1995–2001

Gabriela Avilés,* Maria Valeria Paz,* Griselda Rangeon,† Marie Y. Ranaivoarisoa,‡ Nora Verzeri,§ Sandra Roginski,¶ Pablo Baroni,* and Delia Enria*

Local transmission of dengue fever virus in Argentina is increased by the presence of *Aedes aegypti* mosquitoes and dengue outbreaks in neighboring countries. From 1995 to 2001, a laboratory-based active surveillance program detected 922 dengue cases. Indigenous transmission involving dengue-1 and -2 serotypes was confirmed only in subtropical areas in northern Argentina.

The mosquito *Aedes aegypti*, which carries dengue fever virus (DENV), reinfested Argentina in 1986 and is well established in the subtropical and temperate areas of the country (1); DENV infection was first diagnosed in the laboratory in Argentina in 1997 (2). Early evidence of sporadic DENV-2 circulation was detected in northwestern Argentina in 1997 in a subtropical area; 19 of 404 indigenous cases were confirmed (2). Dengue surveillance in Argentina was organized as a collaboration of the National Department of Epidemiology, National Ministry of Health, provincial departments of epidemiology, national and provincial programs of vector control, and DENV laboratory network. (The DENV laboratory network is coordinated by the Instituto Nacional de Enfermedades Virales Humanas "Dr. Julio I. Maiztegui" [INEVH], the national reference center of DENV diagnosis in Argentina, and the Pan American Health Organization/World Health Organization [PAHO/WHO] Collaborating Center in Viral Hemorrhagic Fevers and Arboviruses.) This network was established in the area at high risk for DENV in Argentina based on the distribution of *Aedes aegypti* from the north to 35°S, the latitude of Buenos Aires (1). Surveillance of imported cases has also been performed in areas with no *Ae. aegypti* infestation because of travelers from areas with DENV transmission who reported DENV-like symptoms. Laboratory-based surveillance has been closely coordinated between epidemiologic and clinical surveillance and vector control measures. Goals of the laboratory network include providing early information on timing and location of transmission, disease severity, and serotypes and genotypes present; predicting transmission; and guiding implementation of clinical and vector control measures. Actions to alleviate DENV are tailored to each region, with the size of the country, variable geographic characteristics, funding, and size of the population at risk taken into account. These actions include vector control, health education, community participation, adequate garbage handling, and adequate water supply. Although the strategy is coordinated at a national level, many of the actions are decentralized to the provincial and municipal levels.

This system enabled us to detect an outbreak in the same subtropical area in northwestern Argentina several months after the first DENV introduction in 1997. Some ongoing, undetected, transmissions may have occurred because the same DENV serotype was circulating. However, clinical surveillance did not detect cases compatible with DENV during those months, and laboratory results were negative. We believe that this outbreak could represent new activity because continuous transmission was suspected (although not confirmed) in neighboring countries. This area of Argentina has a continuous movement of persons across the borders, and imported cases were diagnosed before the outbreak.

DENV-2 was isolated for the first time in the country during an outbreak that affected only the Salta Province in 1998 (3). All cases identified then were classified as dengue fever by using PAHO/WHO criteria (4). DENV cases caused by DENV-2 had also been diagnosed in Bolivia in 1996 and 1997 (5). In 1999 and 2000, an outbreak of at least 27,000 cases of DENV-1 occurred in Paraguay. Evidence suggests that >100,000 cases of DENV-1 may have occurred in the Asuncion District, Paraguay (6). This outbreak spilled over into Argentina, where several cases occurred in the northern part of the country. We summarize the results of 6 years of surveillance and, for the first time, document the circulation of two DENV serotypes in Argentina.

The Study

We defined a notified case as any patient whose illness was considered compatible with DENV by a health professional; samples from these cases were sent to provincial laboratories or directly to the national reference laboratory. Case definitions used for probable and confirmed cases of dengue fever, dengue hemorrhagic fever, and dengue shock syndrome were those proposed by PAHO (4). Because DENV transmission was not endemic in

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Argentina, laboratories checked continuously for the further introduction of the virus. Because the introduction of DENV was relatively recent, the total population infected relatively small, and no continuous transmission was detected, all positive serologic samples obtained at the provincial levels were sent to INEVH to be confirmed by other techniques. If a bigger, confirmed outbreak occurs in the future, we anticipate that a small number of positive samples would be evaluated at INEVH. During interepidemic periods, we conducted laboratory surveillance of febrile syndromes of undetermined etiology. Samples obtained from other surveillance program for diseases that are clinically similar to DENV (e.g., rubella, measles, Argentine hemorrhagic fever, leptospirosis and other illnesses fulfilling criteria for viral hemorrhagic fevers) were also tested for DENV.

Local-level physicians in Argentina were familiarized with the case definitions for DENV syndromes from published guidelines (4). After the discovery of the first occurrence of the virus and the organization of the national DENV surveillance system, the training of local healthcare personnel, including physicians and epidemiologists, was intensified through periodic courses, meetings, and seminars. In small localities, such as those in the north of the country, only a few hospitals exist in each locality, and most of them are government-owned; therefore, all personnel were informed and were willing to participate. In bigger provinces, such as Buenos Aires, the healthcare system is organized into regions, each of which has several public hospitals, but all of these hospitals are supported by a provincial department of epidemiology. Periodic meetings held to inform and train staff were quite efficient. We found no evidence of any physicians unwilling to participate in the system.

The laboratory method used in Argentina has been described in previous reports (2,3,7,8). Briefly, serum samples taken from patients with suspected DENV infection 5 days after onset of symptoms (late acute) were tested weekly during the summer and fall and monthly during the rest of the year at provincial laboratories. The laboratories used a commercial kit (ultramicro enzyme-linked immunosorbent assay capture immunoglobulin [Ig] M dengue test, Instituto Pedro Kouri, Havana, Cuba) or IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA). All positive samples and at least 10% of negative samples were sent to INEVH for quality control and confirmation. When the surveillance system was first initiated, all negative samples were tested at the national reference center. At INEVH, samples were tested by MAC-ELISA, plaque reduction neutralization test (PRNT), hemagglutination inhibition (HI) test, viral isolation in C6/36 cells, and immunofluorescence detection (using monoclonal antibodies obtained from PAHO), or by polymerase chain reaction (PCR) techniques, according to the date of onset. In addition, the capsid/premembrane and envelope/nonstructural protein 1 regions of viral isolates were sequenced to determine the genotypes. Acute-phase serum samples (<5 days after onset of symptoms) were sent directly to INEVH and assayed for viral isolation or PCR; samples >5 days of onset were tested by MAC-ELISA for IgM antibodies. During the convalescent-phase period, a second sample was obtained from patients with positive results for DENV by MAC-ELISA and from most patients with acute-phase samples (with results either positive or negative by viral isolation or PCR); these samples were tested along with the acute-phase samples by using PRNT or HI tests. Serial dilutions of each patient's serum were tested against the four DENV serotypes for 80% PRNT or HI tests. The DENV-1 HAW, DENV-2 NGC, DENV-3 H87, and DENV-4 H241 strains were obtained from the Dengue Branch, Centers for Disease Control and Prevention, San Juan, Puerto Rico. Antigens from mice brains infected with each DENV were prepared at INEVH by using the sucrose-acetone method and used in MAC-ELISA and HI tests. According to the IgG titers by PRNT or HI tests, DENV cases were classified as primary or secondary. Primary cases were indicated by titers <160 in the acute-phase sample (<5 days after onset of symptoms), titers <1,280 in the late acute- or convalescent-phase sample, and low or null cross-reactivity among the different DENV serotypes. Secondary cases were indicated by titers >160 in the acute-phase sample, titers $\geq$2,560 in the late acute- or convalescent-phase sample, and high cross-reactivity among the different DENV serotypes.

A laboratory network including all provinces at risk for DENV (according to *Ae. aegypti* distribution) was established in 1998 (Figure 1). The national reference laboratory, which is self-sufficient for production of key reagents (such as antigens and antiserum), participates in the proficiency tests organized under PAHO/WHO and maintains country-proficiency tests on a continuing basis. Commercial kits were evaluated at the national reference center before being used in national programs. Surveillance for yellow fever, St. Louis encephalitis, West Nile virus, and other flaviviruses were also incorporated into DENV diagnostic protocols.

Thirty laboratories were designated by the National Ministry of Health, the provincial ministries of health, and the local municipalities to integrate the laboratory network (Figure 1). Staff persons from 15 of those regional laboratories were trained on DENV diagnosis at INEVH, and 12 were actively working on DENV serologic surveillance. Courses and rotations were part of the training ongoing since the network of laboratories was organized. These 12 laboratories were evaluated on IgM detection by INEVH

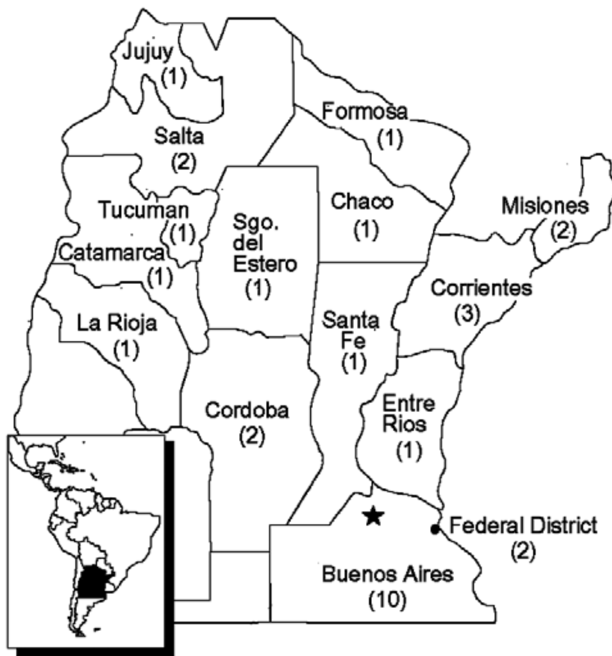

Figure 1. Dengue laboratory network, Argentina. Star indicates the Instituto "Dr. JI Malztegui" (INEVH), Pergamino, National Reference Center ( )= number of local laboratories.

and demonstrated good concordance. The remaining laboratories had difficulties in obtaining reagents, keeping staff because of lack of funding, and other operational problems, so they could not sustain the work through the entire surveillance period. Training was emphasized, and INEVH focused on the problems of these laboratories to maintain high quality control. Staff from all 30 laboratories attended annual meetings at which the results and problems concerning the organization of the laboratory, clinical, and epidemiologic DENV surveillance were discussed. Laboratories sent the samples directly to INEVH when they were unable to carry out sample testing on their own.

During the epidemiologic surveillance of cases compatible with DENV from December 1995 to December 2001, our laboratory received 493 serum samples from travelers returning to Argentina with suspected DENV (Table 1) and from case-patients with no epidemiologic data. Cases were classified as imported or indigenous as a result of epidemiologic analysis, considering travel histories in the 3 weeks before onset of illness. Of 226 positive case-patients, 150 reported travel histories to Paraguay (127 cases), Brazil (11 cases), Honduras (3 cases), Venezuela (3 cases), Ecuador (1 case), Mexico (2 cases), Dominican Republic (1 case), Puerto Rico (1 case), and the Virgin Islands (1 case). Seven other cases were "imported" to other provinces inside the country during the DENV outbreaks of 1998 and 2000. No epidemiologic data were available for the remaining 69 cases. During the DENV outbreak in Salta Province in 1998 (4), 21 persons reported that they had traveled to Bolivia. We could not determine whether infection occurred in Bolivia or Argentina, so these cases were considered as probably imported but were included in the total number of cases (378) for the outbreak (Table 2). Imported cases were detected in different provinces throughout the country in different years: Salta (1996, 2000), Buenos Aires (1997–2000), Santa Fe (1997, 1999, 2000), Misiones (1998, 2000), Jujuy (1998, 2000), Cordoba (1998, 2000), Rio Negro (1998), Tucuman (1999), Chaco (2000), Corrientes (2000), and Formosa (2000); these cases involved either DENV-1, DENV-2, or DENV-3 serotypes (Table 1).

From January 1997 to December 2001, a total of 2,987 serum samples were tested for suspected DENV from provinces in the subtropical (north) and the temperate (central) areas of the country; 696 samples were positive (Table 2, Figure 2). Of these cases, 378 occurred during an outbreak in Salta Province that occurred from January 3 to May 31, 1998, and was caused by DENV-2 (3,7) (Table 2, Figure 2). During the Salta outbreak, men and women

Table 1. Imported dengue virus cases and cases with no epidemiologic data, Argentina, 1995–2001[a]

Y	MAC-ELISA[b]	PRNT[b]	HI[b]	Viral isolates + amplicons[b]	DENV serotype[c]	Total[b] by all techniques
1995	0/18	ND	ND	ND	–	0/18
1996	0/28	ND	ND	1/1	DENV-3	1/28
1997	4/16	2/4	ND	ND	–	4/16
1998	22/64	14/17	ND	ND	DENV-1,2	27/68
1999	17/37	12/12	ND	0/2	DENV-1	23/45
2000	142/307	9/10	43/44	19/31	DENV-1	162/279
2001	9/9	3/3	2/2	ND	–	9/39
Total	194/479	40/46	45/46	20/34	–	226/493[d]

[a]DENV, dengue fever virus; MAC-ELISA, immunoglobulin M antibody capture enzyme-linked immunosorbent assay; PRNT, plaque reduction neutralization test; HI, hemagglutination inhibition test; ND, not done.
[b]Positive/tested.
[c]In most cases, the serotype was determined only by serologic testing unless we indicate otherwise that virus isolation or polymerase chain reaction was also used. In many of these cases, the serotype could not be determined because of cross-reactions in serologic tests.
[d]Some samples were tested by one technique, and others were tested by two or more techniques; therefore, these numbers do not represent the total obtained by adding the rows.

Table 2. Indigenous dengue virus cases in Argentina, 1997–2001[a]

Y	Province	MAC-ELISA[b]	PRNT[b]	HI[b]	Viral isolates + amplicons[b]	DENV serotype [b]	Total[b] by all techniques
1997	Salta	19/387	18/19	ND	1/36	DENV-2	19/404
1998	Salta	359/589	139/143	79/80	5/112	DENV-2	378/701
	Several	0/438	0/56	ND	ND	—	0/457
1999	Salta	5/55	1/1	ND	0/15	—	5/55
	Several	0/171	0/14	ND	0/4	—	0/173
2000	Jujuy	2/63	ND	2/2	2/7	DENV-1	6/72
	Formosa	37/89	1/31	8/21	7/57	DENV-1	50/195
	Misiones	229/442	ND	25/27	9/25	DENV-1	238/469
	Several	0/223	ND	0/19	0/20	—	0/249
2001	Several	0/176	ND	0/2	0/46	—	0/212
Total		651/2,633	159/264	114/151	24/322		696/2,987[c]

[a]DENV, dengue fever virus; MAC-ELISA, immunoglobulin M antibody capture enzyme-linked immunosorbent assay; PRNT, plaque reduction neutralization test; HI, hemagglutination inhibition test; ND, not done.
[b]Positive/tested.
[c]Some samples were tested by only one technique, others were tested by two or more techniques; thus, these numbers do not represent the total obtained by adding the rows.

were equally affected. Prevalence in adults 15–79 years of age (82.5%), was higher compared to that in children <14 years of age (17.5%). Because most samples came from adults, the disease may have been subclinical or undetected in most children. The most affected locality in this region was Tartagal (22°S, 63°W), which had an incidence rate of 67.5 cases per 10,000 inhabitants. Serologic testing by PRNT or HI for all four DENV serotypes in 154 cases from this outbreak resulted in 38 (24.7%) that showed primary responses and 84 (54.5%) that showed secondary responses. The secondary cases were considered to be indigenous because none of these patients had traveled in the previous 3 weeks. We did not gather any information about traveling before those 3 weeks, so whether the patients were infected by another flavivirus in Argentina or in any other country before that is unknown. The remaining 32 cases (20.7%) with borderline IgG titers could not be classified. Previous exposure to other flaviviruses (e.g., St. Louis encephalitis or yellow fever likely due to vaccination) explained 83% of the secondary serologic patterns; the rest remained unexplained, suggesting the unrecognized occurrence of previous infection with other DENV serotypes or flaviviruses other than St. Louis encephalitis or yellow fever (7). The second more important cluster of

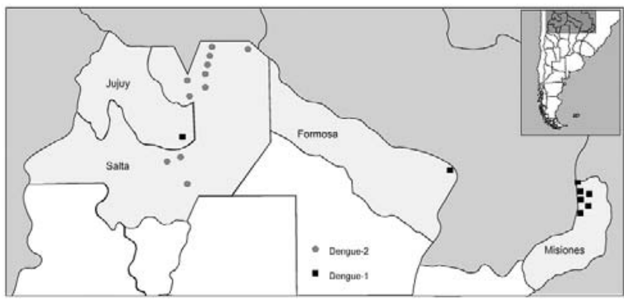

Figure 2. Geographic distribution of dengue cases in Argentina, 1997-2001.

DENV cases occurred during a DENV-1 outbreak in 2000 that affected Misiones, Formosa, and Jujuy Provinces (Table 2, Figure 2), causing 294 cases from February 15 to April 22. Most affected localities were Clorinda (Formosa Province, 25°S, 57°W), with an incidence of 12.3 cases per 10,000 inhabitants and Iguazu (Misiones Province, 25°S, 54°W), with an incidence of 51.7 cases per 10,000 inhabitants.

DENV isolated in Argentina were also characterized molecularly. DENV-2 isolated in Salta in 1997 and 1998 showed 98% to 99% nucleotide identity with DENV-2 isolated from Brazil in 1990 and Jamaica in 1983 (Avilés et al., unpub. data). DENV-1 isolated in 2000 from Misiones, Formosa, and Jujuy Provinces showed 95% identity with a DENV-1 strain isolated from French Guiana in 1989 and 91% identity with a DENV-1 strain isolated from the Caribbean (Jamaica) in 1977 (8).

Conclusions

Laboratory surveillance in Argentina enabled detection of an early circulation of DENV in 1997 and confirmation of two DENV outbreaks that occurred from 1998 to 2000. This surveillance system identified 922 laboratory-diagnosed cases of DENV (696 indigenous, 157 imported, and 69 unknown) during the period of study. We obtained 38 DENV-1, 6 DENV-2, and 1 DENV-3 isolates or PCR amplicons. Imported cases were detected in a wide subtropical and temperate area of the country, while indigenous circulation of DENV occurred only in the subtropical area of the country. The first outbreak caused by DENV-2 occurred in northwestern Argentina in 1998; the second outbreak caused by DENV-1 occurred in the north-northeastern part of the country in 2000. Both outbreaks were preceded by DENV outbreaks in neighboring countries. The rates of DENV infections in neighboring countries varied widely. A serosurvey conducted in Bolivia in 1997

in a neighborhood of Santa Cruz City (10,000 inhabitants) showed that >5% of the populace was infected by DENV-2 (5). Paraguay's Ministry of Health reported an infection rate of 49.3 cases per 10,000 inhabitants (including laboratory-diagnosed and reported cases) during a DENV-1 outbreak in 1999 and 2000 (6). Brazil reported incidence rates in different municipalities in 2000 that varied from 0.037 to 866.9 cases per 10,000 inhabitants (9). The geographic and climatologic situation in Argentina is favorable because most of the country has a temperate climate. Nevertheless, the introduction of different DENV serotypes increases the risk for dengue hemorrhagic fever because of the increased number of secondary DENV infections. The sequence of serotypes in secondary infections seems to be an important risk factor for dengue hemorrhagic fever because DENV-2 secondary infections account for most of those cases. Viral virulence related to specific genotypes is also thought to play an important role (10). All DENV cases that have occurred in Argentina to date have been considered clinically to be dengue fever. DENV-1 that circulated in Argentina during the period of study belongs to an American genotype closely related to viruses circulating previously in other American countries that have been associated mostly with mild disease (8). DENV-2 found in Argentina belongs to an Asian genotype that has been associated with severe disease (11). In Argentina, dengue hemorrhagic fever has not been reported, possibly because the size of the affected population was small and this complication occurs in a small proportion of secondary DENV infections (18–125/1,000) (10), and in an even smaller proportion of primary infections.

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Dr. Avilés established the Arbovirus Laboratory at Instituto Nacional de Enfermedades Virales Humanas, which was designated the National Reference Center for dengue virus diagnosis in Argentina and the Pan American Health Organization /World Health Organization Collaborating Center for Arboviruses; she was chief of the laboratory from 1995 to 2001. She works as an assistant professor at the University of Nevada, Reno, and her research interests focus on molecular epidemiology and pathogenesis of dengue viruses.

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Tick-Borne Encephalitis with Hemorrhagic Syndrome, Novosibirsk Region, Russia, 1999

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Eight fatal cases of tick-borne encephalitis with an unusual hemorrhagic syndrome were identified in 1999 in the Novosibirsk Region, Russia. To study these strains, we sequenced cDNA fragments of protein E gene from six archival formalin-fixed brain samples. Phylogenetic analysis showed tick-borne encephalitis variants clustered with a Far Eastern subtype (homology 94.7%) but not with the Siberian subtype (82%).

Tick-borne encephalitis virus (TBEV) is one of many arthropod-borne viruses from genus *Flavivirus* (family: *Flaviviridae*) pathogenic to humans (1). Infection caused by TBEV is one of the most widespread natural foci infections in Russia; incidence varies from 5,593 to 10,298 cases annually and includes 89–166 deaths (2,3). The incidence of tick-borne encephalitis (TBE) increased sevenfold from 1974 to 1999 in Russia. The geographic distribution of the infection is uneven, with most illnesses occurring in the Siberian and Ural regions. In these regions, incidence is 10 to 30 times higher than in the Russian Far East region, where TBEV was discovered in 1937.

The major surface glycoprotein E is commonly used for studying phylogenetic relations of different TBEV strains. Analysis of the protein E sequence of 16 European and Asian TBEV strains showed clear segregation into three genetic subtypes, designated as European, Far Eastern, and Siberian (4). Genotyping of 75 TBEV strains typical for southern regions of Western Siberia showed that they differ considerably from European and Far Eastern strains (5–7). In reviewing published data, we found no substantial changes in the E gene in the Siberian subtype in 1981 to 1992 (8).

Our study was performed in the Novosibirsk Region of Western Siberia, where 243 to 534 cases of TBE are reported annually. We investigated retrospectively the first reported cases of lethal TBEV infection with hemorrhagic syndrome by using archival histologic samples. To determine the TBEV genotype that probably caused the hemorrhagic form of the infection, we sequenced cDNA fragments of protein E gene. We found that the TBEV strains that most likely caused the infection with hemorrhagic syndrome also carry unique mutations in protein E and belong to the Far Eastern genomic subtype.

The Study

In 1999, a total of 447 TBE cases confirmed by enzyme immunoassay were reported in Novosibirsk Region (Figure 1). Nine (2.0%) patients died; 72.9% of cases occurred in the city of Novosibirsk or its suburbs. The deaths of eight of these patients were associated with a pronounced hemorrhagic syndrome; symptoms included massive gastrointestinal bleedings and multiple hemorrhages in mucosa and internal organs. Four of the patients who died resided in the Toguchin District; the remainder lived in districts located near Novosibirsk (Figure 1). A total of 371 and 358 cases of TBEV infection confirmed by enzyme immunoassay and reverse transcriptase-polymerase chain reaction (RT-PCR) were reported in 2000 and 2001, respectively. In 2001, the mortality rate increased to 3.6%. Surveillance for TBEV during the summers of 2000 and 2001 identified no new cases of TBE with the hemorrhagic syndrome. This lack might be

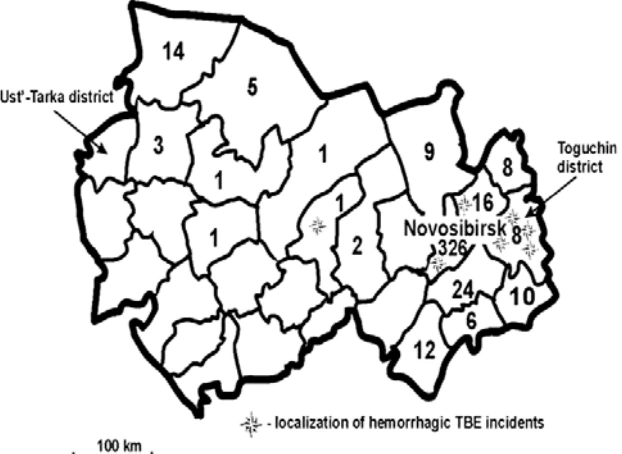

Figure 1. Distribution map of tick-borne encephalitis (TBE) cases by district, Novosibirsk Region, Russia, summer 1999. Case-patients were defined as persons who died from May 1 to August 15, 1999, and who had serologically confirmed (immunoglobulin M–positive test) tick-borne encephalitis infection.

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ascribed to the decrease in TBE incidence during this period, the change in the circulation of TBEV strains, and an unusual weather pattern during May and June.

We found no published reports describing a hemorrhagic disease caused by TBEV, although hemorrhagic manifestation is typical for tick-borne flaviviruses, including Omsk hemorrhagic fever virus (OHFV), Alkhurma virus, and Kyasanur Forest disease virus (9). Cases of OHFV occur occasionally in the Novosibirsk Region. Most cases result from the direct contact of a human with a muskrat, which was introduced in Siberia in 1928 (10,11), and most occur in the Ust' Tarka District (Figure 1), located on the western border of the Novosibirsk Region (12). We found that hemorrhagic TBE emerged in the Toguchin district, located on the eastern border of the Novosibirsk Region, approximately 500 km from the Ust' Tarka District. We found no cases of TBE in the central districts of the Novosibirsk Region, where the lakes and marshes make an unfavorable environment for the spread of this virus. The discovery of hemorrhagic TBE in the eastern part of the Novosibirsk Region is probably not related to the OHFV found in the far western part of the region.

By reviewing all available medical records, we retrospectively analyzed the eight cases of fatal hemorrhagic TBE infection. Diagnoses were confirmed serologically by testing for specific antiviral immunoglobulin M antibodies. All cases occurred in June and July 1999 after patients were bitten by ticks; the latent period was from 5 to 26 days (average 12.8). The ages of the patients ranged from 44 to 69 years. Disease onset included typical TBE clinical symptoms such as fever, myalgia, and malaise, followed by pronounced viral encephalitis accompanied by loss of consciousness, pareses, and paralyses. Hemorrhagic symptoms developed as massive gastrointestinal bleeding and local hemorrhages on mucosa and skin. The first sign of the hemorrhagic syndrome was erythrocytes in urine on day 7 of the infection, which is not usual for TBE infection. Common central nervous system manifestations occurred 3 days later. Patients died 2–3 days after the massive hemorrhagic syndrome developed, despite intensive treatment. The average time of death was 16 days after illness onset. Autopsies showed the pronounced hemorrhagic syndrome and viral encephalitis. Our retrospective screening of medical records from 1999 did not produce any evidence of a milder hemorrhagic syndrome in the rest of the case-patients.

Since hemorrhagic TBE has not been described previously, our objective was to determine the genotype of the virus. However, only six brain tissue samples from the fatal cases were available at the time this study was initiated. Archival samples of formalin-fixed brain tissue from the fatal hemorrhagic cases were collected in March 2000 and stored at −70°C. These samples had been stored for 8 to 9 months in 10% formaldehyde solution at room temperature at the pathology laboratory of the First Municipal Clinical Infectious Hospital of Novosibirsk. Viral RNA was isolated from formalin-fixed brain tissue by using the modified protocol described by Masuda et al. (13) and Coombs et al. (14). The RT-PCR system (GeneAmp RNA PCR Kit, Perkin-Elmer, Branchburg, NJ) was developed for detection of TBEV. RT-PCR primers were designed by using conserved DNA regions encoding gene E of TBEV, strain 205: 5′-TGCACACAYYTGGAAAACAGGGA-3′ (TBE913F), 5′-TGGCCACTTTTCAGGTGGTACTTGGTTCC-3′ (TBE1738R). The sense primer 5′-CAGAGTGATCGAGGCTGGGGYAA-3′ (TBE1192F) and antisense primer 5′-AACACTCCAGTCTGGTCTCCRAGGTTGTA-3′ (1669R) were used for second round of PCR.

Nucleotide sequences of E gene fragment PCR products (1,192–1,661 bp) of TBEV strains were determined by using a Beckman sequencing kit and Beckman CEQ2000XL DNA Analysis System (Beckman Coulter, Inc., Fullerton, CA) according to manufacturer's instructions. We submitted these sequences to GenBank (accession nos. AF540029–AF540034).

The homology values between the nucleotide sequences of protein E fragment of hemorrhagic TBEV, Siberian, and Far Eastern TBEV subtypes are shown in Table 1. The nucleotide sequences of hemorrhagic TBEV strains show approximately the same degree of homology (82%) with protein E gene of different strains of Siberian subtype (strains Lesopark-11, Eltsovka-2, and Vasilchnenko isolated near Novosibirsk) and 94% homology with the Far Eastern subtype. The typical phylogenetic tree with support values is shown in Figure 2. Because hemorrhagic TBEV strains clustered with the Far Eastern subtype of TBEV, we associated them with the Far Eastern subtype.

The amino acid sequence of hemorrhagic TBE has higher homology with the Far Eastern subtype (98%) than with the Siberian subtype (96%). We identified 13 different amino acid mutations in six sequenced fragments of protein E (Table 2). These mutations were not previously described for TBEV; however, most of them (except 121C, 244L, and 249A) were found among other flaviviruses. Cysteine 121 is highly conserved among all flaviviruses since it is involved in maintaining the protein E 3D structure through a disulfide bridge with cysteine 92. Substituting cysteine 121 for glycine in the Koltsovo 31 TBEV variant might cause dramatic changes in protein E structure and function.

Conclusions

Long-term surveillance in Siberia indicates that persons with TBEV infection have relatively mild fever; death occurs in 1% of patients (2). The high incidence of TBE in

Table 1. Homology between the nucleotide acid sequences of prototype strains for three subtypes of tick-borne encephalitis virus, Omsk hemorrhagic fever virus, and hemorrhagic tick-borne encephalitis virus[a]

Viruses	European subtype (subtype 1) (%)		Far Eastern subtype (subtype 2) (%)			Siberian subtype (subtype 3) (%)			Omsk hemorrhagic fever virus
	Neud	KemI	Sofiin	Oshima1	Crimea	Botsad	Eltsovka	Lesopark	Omskhf
Neud	100.00	98.06	81.94	82.78	82.50	84.72	83.89	84.72	80.28
KemI	98.06	100.00	81.39	82.50	81.94	83.89	83.06	83.89	80.00
Sofiin	81.94	81.39	100.00	95.83	94.44	85.28	84.72	85.28	77.78
Oshima1	82.78	82.50	95.83	100.00	97.50	85.83	85.28	85.83	77.22
Crimea	82.50	83.06	94.44	97.50	100.00	86.67	85.56	86.67	78.33
Koltsovo 1	**81.67**	**81.11**	**99.72**	**96.11**	**94.72**	**85.00**	**84.44**	**85.00**	**78.06**
Koltsovo 19	**80.28**	**79.44**	**92.50**	**93.61**	**93.06**	**83.33**	**82.22**	**83.33**	**76.67**
Koltsovo 23	**78.61**	**78.06**	**91.39**	**92.22**	**91.94**	**82.50**	**81.39**	**82.50**	**75.00**
Koltsovo 29	**80.83**	**80.00**	**92.78**	**93.89**	**93.33**	**83.61**	**82.50**	**83.61**	**77.50**
Botsad	84.72	83.89	85.28	85.83	86.67	100.00	97.78	98.89	78.89
Eltsovka	83.89	81.94	84.72	85.28	85.56	97.78	100.00	97.78	78.06
Lesopark	84.72	83.89	85.28	85.83	86.67	98.89	97.78	100.00	78.06
Omskhf	80.28	80.00	77.78	77.22	78.33	78.89	78.06	78.06	100.00

[a]Viruses shown in boldface are the hemorrhagic variants of tick-borne encephalitis virus.

Western Siberia is attributed to the active circulation of Siberian TBEV variants (3,6,7). By sequencing a fragment of protein E, they found these variants grouped to a separate subtype (Siberian). Overall, 75 protein E sequences of TBEV variants isolated in 1952 to 2002 in different regions of Russia were published. Of these virus isolates, 46 were collected directly in the suburbs of Novosibirsk (8). The study showed that Siberian variants have approximately 78% to 81% homology in protein E genes with the Far Eastern TBEV subtype.

Our sequencing data showed that the genome of hemorrhagic TBEV variants differs from already known Siberian TBEV and OHFV strains. Variants of hemorrhagic TBEV show the highest degree of homology with the Far Eastern subtype, represented by prototype strains 205 and Sofiin. This finding supports our hypothesis that a relationship exists between the occurrence of unusual clinical disease and emergence of new TBEV variants in the Novosibirsk Region.

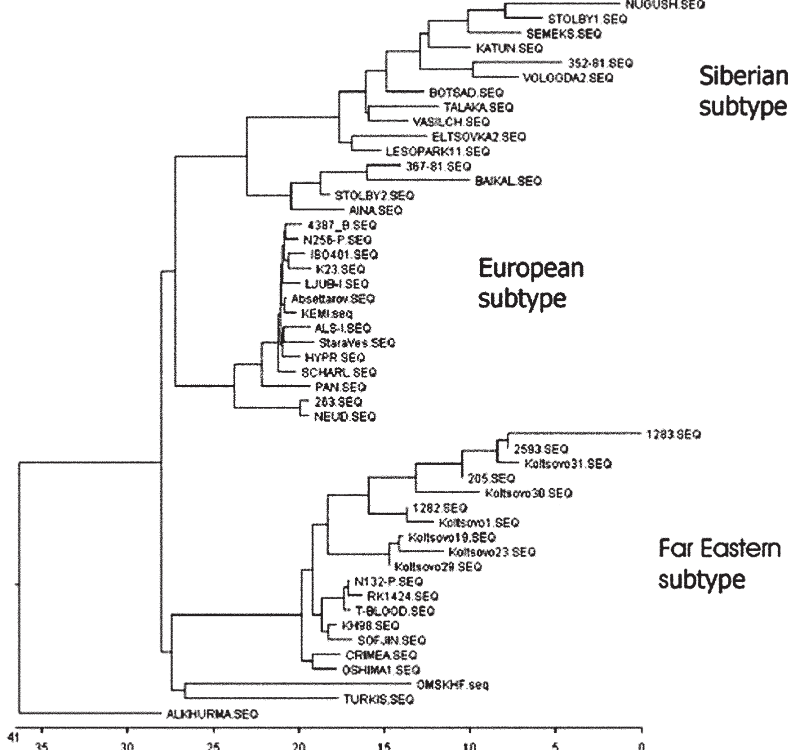

Figure 2. Phylogenetic tree illustrating the genetic relationship of hemorrhagic tick-borne encephalitis virus variants with prototype strains from other subtypes of the virus (generated for nucleotide sequences of protein E gene, fragment 1,192-1,669). Nucleotide and deduced amino acid sequences were aligned by using Clustal X and MEGALIGN v4.04. Phylogenetic tree was constructed by MEGA v2.1. GenBank data were used for comparison of strains of tick-borne encephalitis viruses with another tick-borne flaviviruses. The list of the sequences used (locus name and accession no.) is: 263, 4387/B7, 973, Absettarov, Als.I, Hypr, Iso 40, K23, Kem I, Ljub.I, N256, Neudoerfl, Pan, Scharl, Stara Ves, Turkish, Western subtype Mandl (U27491, X76608, AF241774, AF091005, AF091007, U39292, AF091009–12, AF091014, U27495, AF091015, AF091017, AF091018, L01265, NC001672); Crimea, Hehcir, KH98-10, N132, O-I-1, Oshima C-1, Oshima-5-10, RK1424, Sofiin, T-blood, Latvia (AF091008, AF229363, AB022297, AF091013, AB022292, AB022294, AB001026, AF091016, X07755, AF091019, AJ010192); 352-81, 367-81, Aina, Baikal-4, s Botsad-1, Katun-3, Nugush-2, Semeks, Stolby-1, Stolby-4, Talakan-4, Vasilchenko, Vologda-2 (AF224667, AF241773, AF091006, AF229362, AF224662, AF236055, AF224663, AF224665, AF224666, AF231807, AF241772, AF069066, AF229364); Omsk hemorrhagic fever virus (Omskhf) (X66694); and Alkhurma virus (NC004355).

Table 2. Mutations in the amino acid sequence of protein E of hemorrhagic variants of TBEV

Amino acid, position	TBEV,205	Mutation in hemorrhagic TBEV	Variants of hemorrhagic TBEV	Mutations in other flaviviruses[b]
121	Cys	Gly	Koltsovo 31	
128	Thr	Ala	Koltsovo 19	Val,[c] Ser,[d] Ala,[e] Ile[f]
129	Gly	Arg	Koltsovo 31	Leu[d]
138	Val	Ala	Koltsovo 19	Thr,[c,g] Gln,[d] Lys,[e,f,h–k] Glu[l,m]
141	Val	Asp	Koltsovo 31	Val,[c,d,g] Glu,[e,f, h,i] Ser,[j] Thr[k,l,m]
170	Glu	Gly	Koltsovo 29	Gly,[d] Pro,[e,f,h–j,m] Ser[k,l]
171	Arg	Lys	Koltsovo 1	Lys,[c,g] Ser,[d,f,h,i,m] Ala,[e] Thr,[j,k] Ile[l]
189	Ala	Ser	Koltsovo 23	Thr,[g] Gln,[d] Arg,[e,f,h–l] Gly[m]
223	Leu	Trp	Koltsovo 23	
224	Ala	His	Koltsovo 23	Ser,[g] Thr,[d] Leu,[e] Asn,[h,i] Pro[j–m]
244	Phe	Cys	Koltsovo 30	
245	Gly	Val	Koltsovo 29	Glu,[d–f,h,i] Lys[j–m]
249	Ala	Arg	Koltsovo 30	

[a]TBEV, tick-borne encephalitis virus.
[b]Alignment of protein E sequences was carried out with data from the Protein Families database (available from: URL: http://www.sanger.ac.uk/Software/Pfam/).
[c]Powassan virus, strain LB (Q04538).
[d]Yellow fever virus, strain 17D (P03314).
[e]Murray valley encephalitis virus (P05769).
[f]Japanese encephalitis virus, strain CH2195 (O09754).
[g]Kyasanur forest disease virus (SWISS-PROT Q82951).
[h]Kunjin virus, strain MRM61C (P14335).
[i]St. Louis encephalitis virus, strain MS1-7 (Q88788).
[j]Dengue virus type 1 (O10246).
[k]Dengue virus type 3 (P27915).
[l]Dengue virus type 2, strain 16681 (O09234).
[m]Dengue virus type 4 (Q88668).

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Poor Potential Coverage for 7-Valent Pneumococcal Conjugate Vaccine, Malawi

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Streptococcus pneumoniae infections can be prevented by using new conjugate vaccines, but these vaccines have limited serogroup coverage. We report the first serogrouping data from carried and invasive isolates obtained from children and adults in Malawi. The 7-valent vaccine would cover 41% of invasive isolates from children and 25% from adults. A 9-valent vaccine, including types 1 and 5, would cover 66% of invasive isolates from children and 55% from adults.

Streptococcus pneumoniae is the leading cause of bacterial meningitis in both children and adults in Malawi, as well as an important cause of pneumonia, bacteremia, and death for all ages (1,2). The incidence of *S. pneumoniae* was reduced in the United States and Europe after the licensing of a 7-valent pneumococcal conjugate vaccine (3), and the Global Alliance for Vaccines and Immunization (available from: URL: http://www.vaccinealliance.org) has plans to expand the use of the vaccine to sub-Saharan Africa (4). However, few data from the central African region exist on which to base a pneumococcal conjugate vaccination program.

Methods

Collection of Pneumococcal Isolates

Queen Elizabeth Central Hospital, the main referral hospital for southern Malawi, admits approximately 12,000 children and 10,000 adults per year. From 1996 to 1998, blood cultures and cerebrospinal fluid (CSF) samples were taken from patients admitted to emergency in whom they were indicated. Guidelines suggest that blood cultures should be taken from all febrile patients and lumbar puncture performed if two of three clinical findings (headache, fever, and altered consciousness) indicate meningitis. *S. pneumoniae* were identified from these samples by using colony morphology on blood agar, optochin sensitivity, and Gram stain. All *S. pneumoniae* isolates collected from these samples were stored in bead and broth bacterial cryopreservers (Prolab Diagnostics, Ontario, Canada) at –80°C for serogrouping at a later date.

Carriage Study

From March to July 1998, we collected samples from 250 children and 500 adults by using a single posterior nasal swab, with a sterile cotton swab dipped in saline. The swab was then directly rolled on to a sheep blood agar plate and incubated overnight. Colony subcultures with typical morphologic findings were placed on a second blood agar plate with an optochin disc and cultured at 35°C and 5% CO_2. After 18–24 hours, colonies selected on the basis of typical colony morphologic findings and Optochin sensitivity were stored at –80°C.

Serogrouping

Stored isolates were recultured for typing by using both sheep blood agar plates and enrichment broth (brain heart infusion and Vitox [Oxoid, Basingstoke, U.K.]). Pneumococcal serogrouping was performed with the Quellung reaction with a standard technique (5) and a diagnostic kit from Statens Serum Institut (SSI), Copenhagen, Denmark. This diagnostic kit uses a matrix to group pneumococci covered by the 23-valent pneumococcal vaccine but does not allow typing of pneumococci to the 90 described capsular types. All serogrouping was performed by two investigators (S.B.G. was trained at the World Health Organization [WHO] Pneumococcal Reference Laboratory at SSI, Copenhagen; he trained S.K. who sent a set of serogrouped isolates and isolates that could not be typed by kit to SSI for confirmation of accurate technique).

Results

A total of 628 invasive pneumococcal isolates were collected from the emergency pediatric and medical service. These isolates consisted of 114 pediatric blood cultures, 206 pediatric CSF samples, 208 adult blood cultures, and 100 adult CSF samples. In the carriage study, 105 isolates were collected from 250 children (42% carriage) and 54 isolates were collected from 500 adults (10.8% carriage). Because of a storage accident, some isolates (predominantly pediatric CSF samples) thawed and were not recovered. The total isolates available for serogrouping was 428 (Figure).

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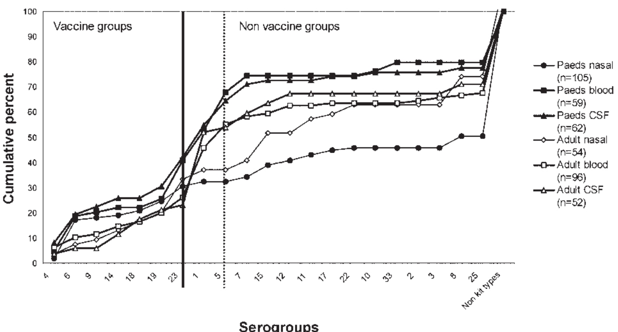

Figure. The cumulative percentage of all pneumococcal isolates plotted by source (see key). Serogroups covered by the 7-valent vaccine are plotted to the left of the heavy vertical line, and the potential 9-valent coverage is illustrated by the dotted vertical line.

The serogrouping results are summarized in the Figure. Types 1 and 5 accounted for 27% and 23% of the isolates from pediatric blood and CSF samples, respectively, and 29% and 31% of the isolates from adult blood and CSF samples, respectively. The potential coverage of the 7-valent conjugate vaccine, which excludes these types, was poor. A high percentage of non–23-valent vaccine type pneumococci existed in adult invasive isolates (35%). A sample of 10 such isolates was sent to the WHO Pneumococcal Reference Laboratory for confirmation of serogrouping. Five strains could not be typed or were acapsulate ("rough") pneumococci, three were not vaccine strains (28F, 16F, and 31), one was *S. oralis,* and one was a misclassified type 7C.

Discussion

These data are the only recent pneumococcal serogrouping data from children and adults available in this region of Africa. As expected, the data showed high rates of pneumococcal carriage in children and lower rates in adults. The pattern of serogroups showed higher rates of types 1 and 5 than that reported from the United States and Europe. The pattern seen in Malawi is similar to that from West Africa (6). A higher incidence of non–23-valent vaccine type pneumococci occurred in our study compared with the incidence in other studies, particularly in our adult isolates; this finding may reflect the high incidence of HIV seropositivity in our population. Recent data from South Africa indicate an increase of typical pediatric carried types (non–23-valent types), causing disease in HIV-infected adults (7). Other recent data are available from coastal Kenya (8), but both of these datasets represent a different population, in terms of ethnic diversity, geographic separation, and HIV seroprevalence.

The potential coverage of invasive pneumococcal isolates offered by the available 7-valent vaccine was poor in Malawi. Previous estimates have suggested that conjugate vaccine coverage in this region would be 70% to 88% (9). We tested a large number of isolates; although some were lost before serogrouping could be performed, the lost isolates probably did not alter the serogroup distribution in our study. In 1980 before the 23-valent polysaccharide vaccine was formulated, serogroups 1 and 5 were considered essential in vaccines for use in Africa (6); a 9-valent vaccine, including serogroups 1 and 5, is already under trial in the Gambia and South Africa (4). Our study indicates that the inclusion of types 1 and 5 will make a substantial difference to the efficacy of the vaccine in Malawi. Alternatively, protein vaccines may have high efficacy against a still wider range of pneumococci.

Antibiotic use is very low in Malawi compared with other parts of the world, and *Pneumocystis carinii* pneumonia prophylaxis is almost unknown because of the lack of healthcare provision. Very low rates of pneumococcal resistance to penicillin (14%), chloramphenicol (24%), and erythromycin (1%) exist in Malawi (10). However, resistance to co-trimoxazole is high (94%) because of the use of sulphamethoxazole-pryrimethamine as firstline anti-malarial drug therapies (11). We were not able to compare the incidence of different serotypes between resistant and nonresistant strains because of the small numbers of penicillin-, chloramphenicol-, or erythromycin-resistant isolates and the small number of cotrimoxazole-sensitive isolates.

HIV infection is a particular problem in Malawi, both in adults and children. An estimated 18% of pediatric hospital patients, 72% of adult hospital patients, and 95% of adults with invasive pneumococcal disease are seropositive for HIV in Blantyre, Malawi (12). Conjugate vaccine is safe and immunogenic in HIV-infected persons (13), and conjugate vaccine was shown to be effective in HIV-infected children, albeit at lower levels than in non–HIV-infected children (14). The vaccine coverage of pneumococcal types, vaccine percentage efficacy, and stage of HIV disease in vaccine recipients are critical determinants of whether this strategy will be of value in adults. However, exposure to children carrying pneumococci is a known risk factor for invasive pneumococcal disease in adults (4). Therefore, reduced carriage of disease-causing pneumococcal strains in children could reduce the adult incidence of invasive pneumococcal disease in this population. Pneumococcal serogroups will continue to be monitored in disease-causing isolates so that vaccine can be implemented in a timely manner.

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Imported West Nile Virus Infection in Europe

To the Editor: We report the case of an 82-year-old man, recently arrived in France from Atlanta, Georgia, USA, who had chills and fever in late August 2002. He left Atlanta on August 26, 2002, and spent a week in Paris before he reached Burgundy. On day 9, after his arrival in the town of Dijon, he had chills and fever (40°C), weakness, malaise, diarrhea, and headache. He was then admitted to Dijon University Hospital. Physical examination indicated hyporeflexia, mild changes in mental status, and no neck stiffness. No lumbar puncture was performed.

Laboratory findings included the following: hyponatremia, 129 mmol/L (normal range 135–145 mmol/L); C-reactive protein, 13 mg/L (normal <3 mg/L); lymphocyte count, 500 cells/mm^3 (normal range, 1,000–4,000 cells/mm^3); positive antinuclear antibodies, 1/160 (homogenous); and positive anti-DNA antibodies, 76 Word Health Organization (WHO) U (normal <39 WHO U). Blood and urine samples remained sterile. Results of the chest roentgenogram and electrocardiogram were normal.

Serum immunoglobulin (Ig) M antibodies to West Nile virus (WNV) were detected by using antibody-capture enzyme-linked immunosorbent assay (ELISA); IgG antibodies were not found by ELISA. A second serologic test performed 2 weeks later in the United States also showed specific IgM antibodies to WNV. The diagnosis of WNV infection was thus established. Four days after admission, the patient no longer had a fever, and his mental status was normal.

WNV infection is a potentially lethal mosquito-borne infection (1). Since 1994, many notable outbreaks have occurred (2–4). The virus emerged in New York, New York, USA, in 1999, and WNV infection is likely to become enzootic in the United States (5). In 2002, WNV was reported in 43 states, and human cases were reported in 33 states (6). The first human case in Georgia was described in 2001.

We report the first imported case of WNV infection in Europe, on the basis of criteria established by the Centers for Disease Control and Prevention. WNV infection was suspected because our patient arrived from an area where WNV is epidemic during the late summer. Clinical findings were similar to those described in previous cases (5). Encephalitis was suspected because the patient showed a reversible alteration of mental status and headache. Although hyponatremia and lymphocytopenia have previously been reported in cases of WNV infection, positive anti-DNA antibodies is a finding of particular interest. However, the mild elevation of antibody titers is common in other viral infections, especially in those caused by members of the *Flaviviridae* family such as hepatitis C virus (7). Although the prognosis of WNV infection is generally poor in elderly patients, our patient had a good outcome (5,8).

In conclusion, physicians in western Europe should be aware of the risk of WNV infection among travelers from a disease-endemic area such as the United States, especially during late summer. Specific antibody detection tests should be performed in such patients with unexplained fever, particularly when they show evidence of neurologic disease. Suspected and confirmed cases can then be quickly reported to health departments, leading to an improvement in the public health response. However, imported cases like this one are not likely to contribute to the spread of WNV infection in Europe. Indeed, human viremia levels seem too low and of insufficient duration to allow the infection of competent mosquito vectors and the subsequent transmission of the virus to other hosts, such as horses or humans (9).

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Rift Valley Fever Virus Infection among French Troops in Chad

To the Editor: During the rainy season every year, outbreaks of self-limiting nonmalarious febrile syndromes have occurred in French military troops on duty in Chad. To determine the cause of these syndromes, the Tropical Medicine Institute of the French Army Medical Corps implemented an arbo-virus surveillance program in Marseille.

During summer 2001, we collected samples from 50 soldiers who had a febrile illness. All blood spot samples tested negative by enzyme-linked immunosorbent assay (ELISA) for certain antigens (i.e., dengue virus, West Nile virus, Chikungunya virus, and Wesselsbron virus). However, after co-culture of 31 peripheral blood lymphocyte samples with C6/36 and Vero cell lines collected in NDjamena, Chad, in August to September 2001, two strains of Rift Valley fever virus (RVFV) were isolated and identified by using indirect immunofluorescence with a specific mouse ascitic fluid and by using reverse transcriptase-polymerase chain reaction (RT-PCR) and sequencing. In retrospective testing, we found that all serum specimens tested by ELISA for RVFV-specific immunoglobulin (Ig) M and IgG were negative. The second serum samples from the two case-patients with these strains, collected 2 months later, were strongly positive (IgM 1/200,000; IgG 1/5,000).

Rift Valley fever, a febrile disease that affects livestock and humans, is transmitted by mosquitoes and caused by a virus (genus: *Phlebovirus,* family: *Bunyaviridae*) that can persist in nature in contaminated eggs. The virus was first isolated in Kenya in 1930 (1) and is endemic in the region. In Chad, the disease was first reported in 1967 at the same time as in Cameroon (2); no strain was isolated at that time. Since 1977, RVFV infection resulted in 600 deaths in Egypt (3), 300 in Mauritania in 1987 (4), and 200 in Saudi Arabia and Yemen (5,6) in 2000 to 2001.

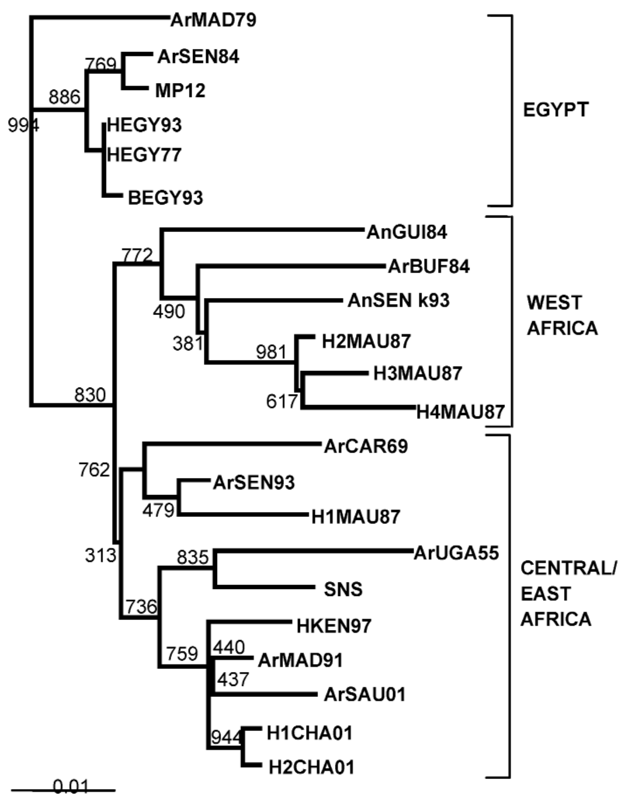

Figure. NSs-based phylogenetic tree of Rift Valley fever virus strains. Values indicate the bootstrap support of the nodes. Strains isolated in Chad are designated H1CHA01 and H2CHA01, according to the previous abbreviation guidelines (7,8). EMBL accession nos. AJ504997 and AJ504998. SNS, Smithburn strain. Branch lengths are proportional to the number of substitutions per site.

To characterize these RVFV strains, parts of the three genome segments (L, M, and S) were amplified by using RT-PCR and sequenced as described (7,8). The figure shows the phylogenic tree constructed from the sequence of the region coding for NSs in the S segment, by using the neighbor-joining method implemented in Clustal W (version 1.6; available from: URL: http://www-igbmc.u-strasbg.fr/BioInfo/ClustalW/clustalw.html). The two strains identified in Chad are quite similar. They are located within the East/Central lineage established previously (6,7), which contains the virus that circulated in Madagascar (1991), Kenya (1997–1998), and Yemen and Saudi Arabia (2000–2001) (9,10). Sequencing of the region in the M and L segments led to the same clustering (not shown), suggesting that this virus did not evolve by reassortment. Deter-mining the origin of the virus is difficult, but its genetic properties suggest that this strain has a Kenyan origin. Before this isolation, no RVFV strains from Chad had been genetically characterized. This strain may be endemic in this region of Central Africa, or the RVFV strain circulating in the Eastern countries may have been transported outside of the territory (which was likely the case in Yemen and Saudi Arabia in

2000) (9,10). Of the two case-patients, one soldier did not leave NDjamena during his 3-month tour of duty, whereas the other had been in contact with livestock in a flooded area before onset of symptoms. Contamination may have occurred through infected animals or mosquitoes, although sheep living in the area did not show any sign of disease (i.e., spontaneous abortions, deaths). The two cases we describe were self-limiting; however, deaths from this illness have been reported in nonepidemic settings in Central African Republic (11). Our data emphasize that healthcare providers should systematically consider Rift Valley fever as a diagnosis for febrile syndromes in persons returning from Africa, even in nonepidemic settings (12).

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Corynebacterium ulcerans Diphtheria in Japan

To the Editor: *Corynebacterium ulcerans* causes a zoonotic infection similar to diphtheria, which is caused by *C. diphtheriae*. Studies indicate that signs and symptoms of a diphtheria-like illness caused by *C. ulcerans* are milder than those caused by *C. diphtheriae*. However, some strains of *C. ulcerans* produce potent diphtheria toxin and may cause severe symptoms similar to those caused by *C. diphtheriae* (1). We report a case of a diphtheria-like illness caused by *C. ulcerans* infection.

A previously healthy 52-year-old woman first noticed hoarseness approximately 3 days before admission to the hospital. On February 16, 2001, severe dyspnea and fever developed, and the patient was referred to the emergency room of the Asahi General Hospital by her private practitioner. Physical examination indicated a large stridor, which could be heard without using a stethoscope. Cyanosis was not observed. The endoscopic examination showed a thick white coat covering the nasopharynx and laryngeal vestibulum, and subglottic constriction was also observed. A chest x-ray showed diffuse infiltrates in both lungs. Pertinent laboratory findings on admission included leukocyte count of 16.8 x 10^3/µL and C-reactive protein of 20.0 mg/dL. The serum level of liver transaminase was normal, and both Wassermann reaction and anti-HIV antibody tests were negative. Pharyngolaryngitis and pneumonia was diagnosed in the patient. Because of severe dyspnea, intubation was performed, which caused sudden and unexpected exacerbation of the condition. Severe cyanosis subsequently developed. Extubation was immediately performed, and a thick white material was found to be filling the lumen of the endotracheal tube. Reintubation was performed, and dyspnea subsided. The patient was hospitalized in the intensive-care unit. Sulbactam sodium/ampicillin sodium (6 g per day) was intravenously administered for 4 days; however, the symptoms were not much improved. The symptoms were most consistent with those of diphtheria. Therefore, the patient was subsequently placed

on erythromycin (1.0 g/day) and quickly responded to this treatment without administration of diphtheria antiserum. Erythromycin was intravenously administered at 1 g per day for 9 days, then orally administered at 1,200 mg per day for the next 14 days. Throughout the hospitalization, no complication occurred, and no abnormalities were noted in the electrocardiograms or in the patient's neurologic status. The patient was discharged uneventfully, and no serious sequelae were noted for 20 months. History of immunization for diphtheria was not known.

After the hospitalization for this acute illness, a laboratory report showed that *C. ulcerans* was cultured from the thick white coat of the throat. No other bacteria were found. The National Institute of Infectious Diseases in Tokyo later confirmed identification of the bacteria. By using Elek's test, Vero cell toxicity, and polymerase chain reaction for toxigene, this strain of *C. ulcerans* was proven to produce diphtheria toxin identical to *C. diphtheriae* (2–4). Although administering appropriate antibiotics as well as antitoxin is a standard of care for patients with diphtheria, antitoxin was not given to this patient because of her rapid response to the erythromycin therapy.

C. ulcerans infections in humans occur after drinking unpasteurized milk or coming in contact with dairy animals or their waste (5,6). However, person-to-person transmission of *C. ulcerans* has not been reported, and in some cases, the route of transmission is not clear (7). Recently, *C. ulcerans*-producing diphtheria toxin was isolated in the United Kingdom from cats with nasal discharge (8).

Our patient did not have direct contact with dairy livestock or unpasteurized dairy products; however, more than 10 dairy farms are scattered around her home. Moreover, she kept nearly 20 cats in her house and had been scratched by a stray cat a week before illness developed. This stray cat, which had rhinorrhea and sneezing, had wandered into her house. The stray cat died before the patient became ill, and no further investigation could be made. Stray cats might well be one of the possible carriers of *C. ulcerans* and might have transmitted the bacteria to this patient. To our knowledge, a case of human infection caused by *C. ulcerans* has never been reported in Japan. On the basis of current experience, this bacterium does exist in Japan and can potentially cause a serious diphtheria-like illness in humans.

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Salmonella in Birds Migrating through Sweden

To the Editor: To determine how common *Salmonella* infection is in the migrating wild bird population, we considered the biology of the bacterium and that of its avian hosts. Previous studies have attempted to determine in which stages wild birds become infected, how infections are acquired, and how this information should be translated into epidemiologic risk assessments for human and animal health. For instance, most published studies originate from small epizootics and are of either dead birds at feeding stations (1) or infected birds in or around barns where the livestock has *Salmonella* infection (2). This bias has important consequences, as the natural prevalence of *Salmonella* in the non-epizootic situation likely is overestimated. Finding infected birds close to a barn with infected cattle does not prove that transmission occurred from the birds to the animals. In addition, an epizootic at a feeding station does not prove that *Salmonella* normally occurs in the inflicted bird species, as the birds could have became infected through proximity to the infected animals, or in the case of the bird feeder, through feed contaminated from an unknown source. We need baseline surveillance data on the prevalence of *Salmonella* in non-epizootic situations, in healthy bird communities and in different

stages of a bird's life (e.g., during breeding, molting, and migration), to better understand *Salmonella* epidemiology in relation to wild birds.

We focused on the migratory bird fauna of the North Western Palearctic, where most of the birds migrate south to spend the nonbreeding season in continental Europe and Africa. In these areas, certain species, such as gulls, corvids, starlings, and thrushes, may overwinter in agricultural and urban areas were domestic animals are present. We sampled apparently healthy birds trapped on active migration at Ottenby Bird Observatory (56°12'N, 16°24'E), on the southernmost tip of the island Öland, southeast Sweden, during the migration periods July–November 2001, March–May 2002, and July–December 2002. We used a standardized trapping and sampling scheme, previously used at the same site for large-scale screening of *Campylobacter* infections in wild birds (3). To apply a random procedure in selection of species and persons, every 10th bird banded during the migration periods was sampled for *Salmonella* spp. We did not sample recaptured birds previously banded by us.

In total, 2,377 samples from 110 species of migratory birds (1,086 samples in autumn 2001, 777 in spring 2002, and 514 in autumn 2002) were analyzed for *Salmonella* infections. We applied routine procedures for isolation of putative *Salmonella* isolates, with enrichment in Rappaport-Vassiliadis broth and injection into xylose-xysine-desoxycholate (XLD) agar. On this media, most *Salmonella enterica* serotype Enterica appears as red transparent colonies with black centers. Colonies with growth characteristics of *Salmonella* were observed in 236 samples, and full phenotypic identification was performed on these isolates by using standard biochemical and serologic testing. By using the API system (4), the isolates were identified as *Citrobacter youngae*, *C. braakii, C. freundii, Escherichia vulneris, E. coli, Hafnia alvei, Klebsiella pneumoniae ozaenae, Acinetobacter baumanii , Providencia stuartii/ rettgeri*, and *Yersinia kristen senii.* Only one of the isolates, obtained from a Mistle Thrush (*Turdus viscivorus*) and sampled during the spring migration 2002, carried *Salmonella.* This isolate was characterized by serotyping according to the Kauffman-White serotyping scheme (5) at the reference laboratory of the Swedish Institute for Infectious Disease Control. The thrush isolate was identified as *S.* Schleissheim, a rare *Salmonella* serotype. Human salmonellosis caused by this serotype has been previously reported only in Turkey (6). No reservoir of *S.* Schleissheim, in animals or in humans, has been reported in Sweden in the last 10 years covered by the current epidemiologic records.

The failure to find *Salmonella* was probably not caused by technical problems. The sampling methods used, with fecal samples from fresh droppings or cloacal swabs, are well-established techniques for studying *Salmonella* prevalence in birds (2,7,8). The laboratory methods used, with enrichment in Rappaport-Vassaliadis broth and subsequent culturing on XLD-agar, are extremely sensitive for detecting *Salmonella*, even for samples highly contaminated with other *Enterobacteriaceae* (9). Thus, in this large dataset, only one *Salmonella* isolate was found, representing a serotype rarely observed in clinical or veterinary samples. In particular, one serotype, *S.* Typhimurium DT40, has been associated with epizootics in wintering passerine birds (10), but this serotype was not found in any of our samples.

Results from our study indicate that the prevalence of this serotype in the healthy wild bird population is low. Our dataset was composed of many different species, but the number of tested individual birds for each species was low in many cases. Earlier studies have pointed to certain species (gulls and corvids) in which the prevalence of *Salmonella* is sometimes high (2% to 20%), and argued that concern should be strong about epidemiologic disease transmission with these birds (7,8). These species have the capability to live in an opportunistic manner in close proximity to humans and can base their diet on waste products and garbage. Most bird species, however, have little or no niche overlap with humans or domesticated animals; virtually no data exists on the occurrence of *Salmonella* in this major group of migrating birds during a non-epizootic situation. Our results suggest that the natural occurrence of *Salmonella* in healthy birds during migration in Sweden may be low. Therefore, the *Salmonella* incidence is probably also low for most wild bird species. We suggest that researchers consider analyzing the non-epizootic natural occurrence of *Salmonella* in wild birds. Accumulated knowledge from many different regions, over many years, is a prerequisite for thorough risk assessment of the importance of *Salmonella* carriage in wild birds.

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Parachlamydiaceae as Rare Agents of Pneumonia

To the Editor: Members of the *Parachlamydiaceae* family are emerging intracellular bacteria living in amoebae (1,2). Serologic studies have suggested that *Parachlamydia acanthamoeba* might be an agent of community-acquired pneumonia transmitted from a water source (3,4). In a single occasion, 16s rRNA of a member of the *Parachlamydiaceae* family was amplified and sequenced from a bronchoalveolar lavage sample (5). Thus, to specify the role played by the *Parachlamydiaceae* as agents of lower respiratory tract infection, we developed a real-time polymerase chain reaction (PCR) assay and applied it to 1,200 bronchoalveolar lavage samples, taken mainly from patients with pneumonia of unknown cause and received in our diagnostic microbiology laboratory between 1997 and 2002.

DNA extraction was performed by using the MagNA Pure LC instrument and the MagNA Pure LC DNA Isolation Kit III (Roche Molecular Biochemicals, Mannheim, Germany). Real-time PCR was performed by using TaqMan technology and targeting the gene encoding for a nonmitochondrial ATP/ADP translocase (GenBank accession no. AF490592). This energy parasite gene is present only in rickettsiae, chlamydiae, and plant plastids (6). The master mixture was prepared from the TaqMan Universal Master Mix kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions, and included 200 nM of each primer (Adp81F 5´- TAGTGATCTGC-TACGGGATTT, Adp84R 5´-TTG-GATTAGGATATTGCAATTT) and 200 nM of the fluorescent labeled probe (6-FAM-5´-AACCTTGTA-GAAGTAACCTGGAA-GAACCAGC-3´-TAMRA, where 6-FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine). Amplification was carried out on the ABI 7700 sequence detection system (TaqMan system, Applied Biosystems), by running 45 cycles, with annealing temperature of 52°C and polymerization temperature of 60°C. To prevent carryover, 200 µ M of uracil triphosphate was part of the master mixture, and uracil-N-glycosylase was used systematically. *Parachlamydia acanthamoeba* strain Hall coccus (kindly provided by T.J. Rowbotham) (3) and sterile water were used as positive and negative controls, respectively. In addition, PCR was tested on *Chlamydophila pneumoniae* and *Chlamydia psitacci* and four strains of *Rickettsia*. All but one (*Rickettsia montana*) was negative, as were 64 sterile water controls.

Of the 1,200 bronchoalveolar lavage samples tested, 5 (0.42%) were positive. When PCR was repeated for those five samples, four were negative for *P. acanthamoeba* DNA, and only one was a true positive, confirmed by sequencing the product of the additional PCR. The sequence shared 100% DNA homology with *P. acanthamoeba* strain Hall coccus (GenBank accession no. AF490592). The patient, a 31-year-old man who was HIV-positive, had pneumonia, cough, and no fever. Chest x-ray examination showed an opacity in the right lung and a bilateral infiltrate. Leukocyte count was 5,000/mm3 with 80 CD4 cells/mm3; microbiologic investigations (in which the bronchoalveolar lavage was examined for cytomegalovirus, *Chlamydophila pneumoniae*, *Legionella pneumophila*, *Pneumocystis carinii*, *mycobacteria*, and *Toxoplasma gondii*) did not identify a causal agent.

We developed a highly sensitive PCR, which could amplify as few as 10 bacteria. The assay results in a relatively high specificity (1,195/ 1,199; 99.67%) because it uses a target gene found only in *Rickettsiae*,

Chlamydiae, and plant plastids, and uses a specific DNA probe. We considerably decreased the risk of horizontal and vertical contamination of the PCR reaction by using uracil and uracil-N-glycosylase and by keeping reaction cups closed since the first amplification cycle.

More importantly, our study showed that *Parachlamydia* DNA is rarely found in bronchoalveolar lavage samples (0.083%). This suggests that persons are infrequently exposed to *Parachlamydia* organisms and, consequently, members of the *Parachlamydiaceae* seldom cause pneumonia in humans. In the only positive sample, whether *Parachlamydia* originated from bacteria in the oropharynx, from water, or from a colonization of the lower respiratory tract was not known; whether they caused the patient's pneumonia is also not known. That two strains of *Parachlamydia* found in amoebae were recovered from the nasopharynx of healthy volunteers (7) favors the first hypothesis. However, that the positive broncholaveolar lavage specimen was taken from an HIV-positive patient with community-acquired pneumonia suggests that *Parachlamydia* might occasionally play a pathogenic role in AIDS patients. Moreover, any amoebae-associated bacteria should be considered as a potential emerging pathogen because intra-amoebal growth may lead to the selection of virulence traits and to the adaptation to professional phagocytes, such as alveolar macrophages (1,2). Further studies are warranted to determine whether *Parachlamydiaceae* causes community-acquired pneumonia, particularly in HIV-infected persons.

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Hantaviruses in the Czech Republic

To the Editor: Infections caused by hantaviruses have been known for a long time, but their causative agent was not detected until 1976 (1). These viruses of the genus *Hantavirus*, family *Bunyaviridae*, have >20 genotypes. Hantaviruses circulate in wild rodents within natural foci over Euroasia and North and South America. They cause asymptomatic persistent infections in these small mammals. Humans may acquire infection accidentally from inhalation of virus-contaminated aerosols of rodent excreta. Hantavirus genotypes may be nonpathogenic for humans or cause serious diseases with high death rates. In Eurasia, these pathogens involve primarily the kidney and cause hemorrhagic fever with renal syndrome; in North and South America, these pathogens involve primarily the lung and cause hantavirus cardiopulmonary syndrome.

First reports on the occurrence of hantaviruses in central Europe originated from former Czechoslovakia (2,3) and Germany (4) and date back to 1984 and 1985, respectively. The first cases of hantavirus disease in humans in the Czech Republic were reported in 1992 (5). This flulike disease accompanied by microhematuria was recorded in southern Moravia. Another severe imported case was described in a soldier on active military duty in the Balkans (6). The first isolation of nonpathogenic hantavirus Tula was reported in the Czech Republic (7). Currently, several hantavirus infections have been recorded in humans, manifesting mainly as interstitial nephritis. One fatal case was also reported in a patient who had never travelled outside the Czech Republic.

We conducted studies of hantavirus ecology in the Czech Republic and hantavirus seroprevalence in the Czech population. As in neighboring Slovakia (8), hantaviruses of three genotypes, i.e., Dobrava, Puumala, and Tula, were identified in the Czech Republic. Most serious infections are caused by the Dobrava genotype; Tula genotype

remains nonpathogenic for humans, although a case of human infection without clinical signs has been described in the Czech Republic (9).

Blood serum samples from 710 randomly selected persons >20 years of age from the Czech Republic were screened for antibodies against Puumala and Hantaan antigens with commercial enzyme-linked immunosorbent assay (ELISA) sets manufactured by PROGEN (Biotechnik, Heidelberg, Germany). The Hantaan antigen was used because of its antigenic relatedness with Dobrava virus, which is not included in available commercial ELISA sets.

Five participants showed immunoglobulin (Ig) G reactivity to Hantaan virus (cross-reactive with Dobrava antigen), and two participants tested positive for both IgG and IgM antibodies. Two other persons showed IgM reactivity alone. These findings indicate that as many as seven (1.0%) study participants showed reactivity to Hantaan antigen. Eight persons showed IgG reactivity to Puumala antigen, none of them IgM positive. Altogether, 10 (1.4%) study participants were reactive to Puumala antigen. Three persons showed reactivity to both antigens tested.

A total of 1,494 small mammals of different Czech regions were screened with ELISA for hantavirus antigen in the lungs. The antigen was detected in the lungs of 101 animals; the highest positivity rate was in Common Voles (Microtus arvalis). The difference in positivity between male and female voles was not statistically significant. The positivity rate was markedly associated with rodent size. With the use of molecular genetic methods (polymerase chain reaction), genotype Tula was identified as the causative agent of infection in rodents. Genotype Puumala was identified in bank voles (*Clethrionomys glareolus*) in Moravia. Nucleotide sequences of Dobrava genotype were identified in southern Bohemian rodents (K. Krivanec, pers. comm.).

In the Czech Republic, Tula virus is the most frequent hantavirus circulating in Common Voles. This agent is not pathogenic for humans. The hantavirus seroprevalence rate in the adult population of the Czech Republic is close to 1%. Dobrava and Puumala viruses are causative agents of these infections in humans.

This research was supported by grants no NI 5896-3/2000 of the Grant Agency of the Ministry of Health of the Czech Republic and no. EVK-CT-2000-00070 of the EC cCASHh.

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Antibiotics and Airline Emergency Medical Kits

To the Editor: Medical events during airline flights have drawn some attention in recently published articles and letters (1–4). We would like to share our experience of meningococcemia/ and meningococcal meningitis during a transatlantic flight.

In June 2000, a 20-year-old student with a mild viral illness (diagnosed before the flight) boarded a flight from Tel-Aviv, Israel, to Newark, New-Jersey, USA (approximate flight time, 11–12 hours), with a tour group of college-age students and their chaperones**.** We, a neonatologist and a neonatal intensive care nurse, were on the same flight to later transport a prematurely born infant from the United States back to Israel.

About 90 minutes before landing in New Jersey, the chief flight attendant asked me (B.B-O.) to check the passenger, who said he did not feel well. His medical history indicated no past illness, which was corroborated by the director of the tour group. The patient reported general malaise and numbness in his feet. In the 2 weeks before the flight, he had traveled in Israel, visiting cities, caves, and mountains. He and his group had slept in different hostels in those areas.

On examination, he was fully conscious, and his blood pressure and

pulse rate were normal. He had a blue-purple skin rash, particularly on the upper extremities. The rash worsened in the course of 20 minutes and resembled the "blueberry muffin–like" rash described in other pathologic conditions. Considering a diagnosis of either tick-borne or meningococcal disease, I decided to give the patient the first dose of antibiotics after obtaining a verbal consent from him and from the head of the group. I also asked the crew to have an ambulance and a physician waiting for us at the destination airport.

When we checked the emergency medical kit, we found that it did not contain any antibiotics. For our transport mission, we had two ampules of cefotaxime, 2 g each, one of which we gave the patient. After we landed, an ambulance crew (which did not include a physician) took the patient to the nearest hospital. The patient died 2 hours later in the hospital emergency department. His laboratory tests showed meningococcal meningitis and meningococcemia. The Centers for Disease Control (CDC), the airline company, and Israel's Ministry of Health notified all close contacts of the patient in Israel and during the transatlantic flight, including everyone in the tour group, and recommended that they be given chemoprophylaxis.

CDC has received 21 reports about air travel–associated meningococcal disease in 2 years; in 5 reports, the symptoms began before the plane arrived at its destination (5). However, advance notice of the symptoms was given only in our case. Although one case is not enough to substantiate recommendations, we believe that the appropriate authorities should require airline companies to add a broad-spectrum antibiotic preparation to the emergency kit. This drug should be used only when aircraft diversion is not possible and when the diagnosis is clinically identified or highly suspected.

We still wonder whether an earlier intervention and treatment with a more appropriate on-board antibiotic treatment would have saved this young man.

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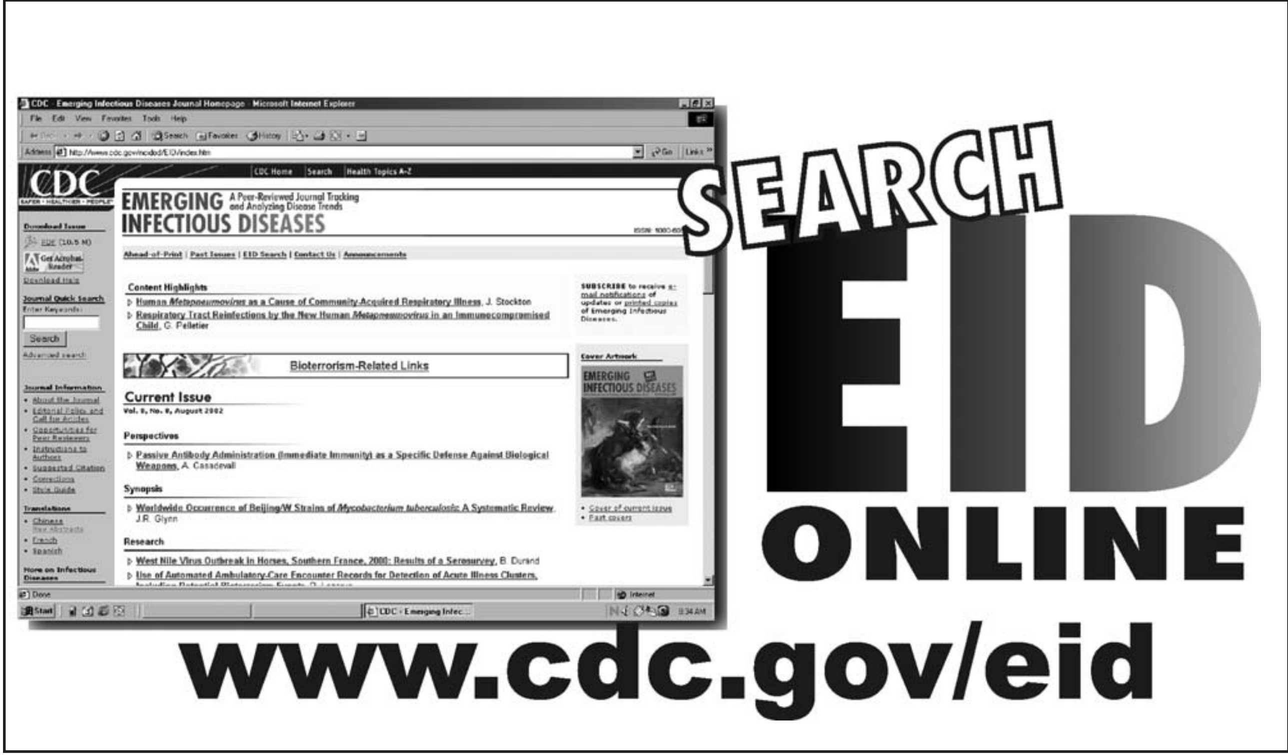

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Pablo Picasso (1881–1973). Guernica (1937). Oil on canvas, 350 cm x 782 cm

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Photo by John Bigelow Taylor/Art Resource, NY

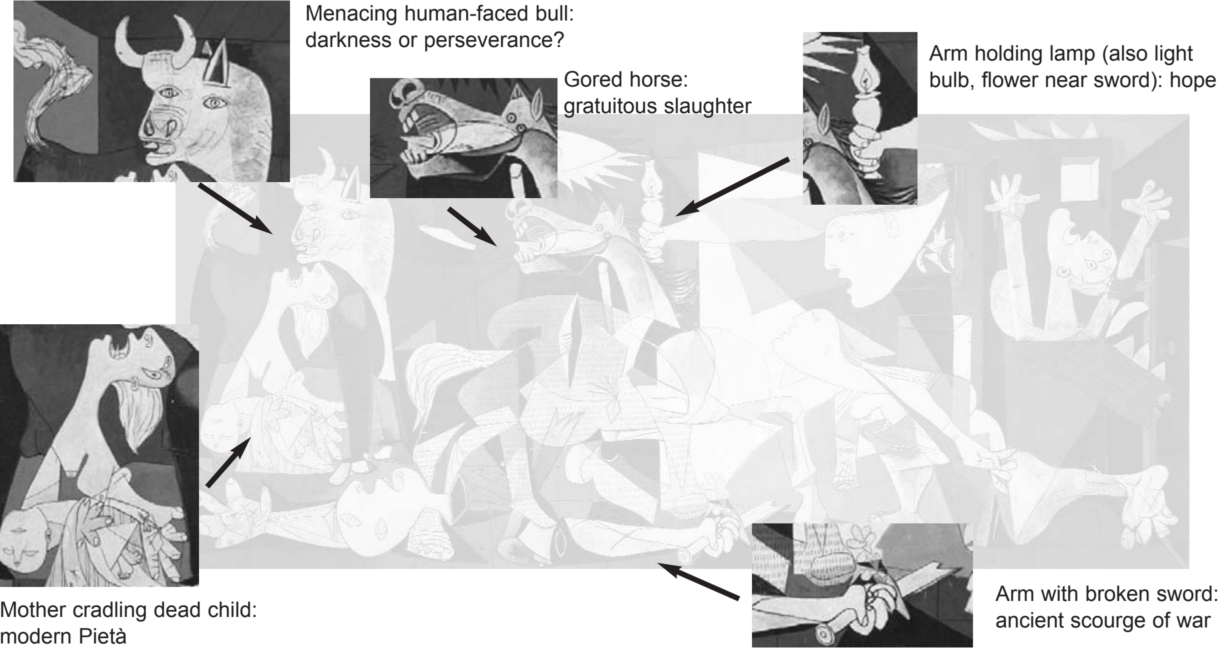
"It isn't up to the painter to define the symbols," said Pablo Picasso when asked to explain his celebrated mural, Guernica. "….The public who look at the picture must interpret the symbols as they understand them." Picasso himself did not know what the work would turn out to be when he was commissioned to paint the centerpiece for the Spanish Pavilion of the 1937 World's Fair in Paris: "A painting is not thought out in advance. While it is being done, it changes as one's thoughts change. And when it's finished, it goes on changing, according to the state of mind of whoever is looking at it" (1,2).

The official theme of the World's Fair was modern technology, but the inspiration for Picasso's mural came from world events—specifically the Nazi bombing and virtual obliteration of Guernica, an ancient Basque town in northern Spain. The bombardment of this nonmilitary target took little more than 3 hours, during which airplanes, plunging low from above the center of town, machine-gunned the townspeople who had taken refuge in the fields (3). News reports and horrific photographs of the massacre quickly reached Paris and provided the story line for perhaps the best-known painting of the 20th century. Started 6 days after the bombing and completed in 5 weeks, the monumental mural captured the agony brought on by brutality and violence.

A native of Andalusia, Spain, who lived most of his life in France, Picasso was the most innovative artist of his era and perhaps any era. His complex genius is usually tracked in a series of overlapping periods beginning in 1901. A master of classical art, he painted the poor, whose ordinary activities he imbued with melancholy and lyricism (blue period, rose period). Around 1905, influenced by Cézanne and African sculpture, he experimented with fragmented and distorted images and became one of the founders of modern abstraction (literally "the drawing away from or separating").

Destroying in order to create, Picasso dismantled traditional forms and sought the inner geometry of objects and the human figure. His Les Demoiselles d'Avignon (1907) marked the beginning of analytic cubism, the harsh intellectual style (also of Braque and Gris) in which decomposition of objects into geometric lines and contours is carried to an extreme (5). Figures and their surroundings are broken into "angular wedges or facets," shaded to appear three-dimensional. We cannot tell if the fragments are concave or convex; some seem "chunks of modified space," others translucent bodies comprising a fantastic world of compounded voids and solids (4).

Traditional art confines its subject to one time and place. Cubism allows the artist to express what Albert Einstein defined in 1905 in his theory of relativity: a new sense of time, space, and energy in which moving figures become an extension of the environment from which they are indistinguishable (4). As art, the world, and self converge, continuity and brokenness, symmetrical progression, life and death, pain and hope can be viewed within a broader aesthetic reality (6). Around 1909, Picasso eliminated color, replacing it with a range of gray and brown



tones to which he added new elements, paper cutouts, numbers, and letters, creating collages and other new techniques that further separated the work of art from any representation of reality. In a later form, cubism became "synthetic," more representational and flat, and included bright decorative patterns (as in The Three Musicians, 1921).

"Art is the lie that tells the truth," Picasso once said, articulating how an abstract painting could pack so much passion. Guernica does not represent the event that inspired it. Rather, in a series of allegorical images, it evokes the complexity and depth of suffering caused by the event. In a systematically crowded composition (deliberately undermining the academic rules of art), figures are crammed into the foreground: screaming mother cradling dead child, corpse with wide open eyes, arm holding lamp, fighter's arm with weapon, menacing human-faced bull, gored horse. In open darkness and surrounded by burning buildings, the figures seem united in a sublime lament, reminiscent of Mediterranean funeral rites (5). Their symbolism defies exact interpretation; they owe their terrific eloquence to "what they are, not what they mean." With flawless internal logic, the anatomical dislocations, fragmentations, and transformations expose the stark reality of unbearable pain (4).

As if conceived during a lightening strike, Guernica shocks in near monochrome, exemplifying the triumph of pure abstracted form. Its symbolism, punctuated by innovative techniques (e.g., inclusion of newsprint), transforms a local event to a universal icon of terror in the aftermath of violence. The figures, human and not, in primitive flight, embody the horror of living creatures under attack. With undeniable clarity, Picasso spells out in modern terms humanity's condemnation of unnecessary suffering, the agony caused not by unavoidable disasters or indecipherable diseases but by unimaginable intentional violence, such as witnessed in the deliberate release of biological agents.

Polyxeni Potter

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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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EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.9, No.7, July, 2003

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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

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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.

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EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends
Vol.9, No.5, May 2003

Hazards of Travel (pg.525)

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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.