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A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Vol.10, No.5, May 2004

The SARS Patient

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

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On the Cover

Francisco José de Goya y Lucientes (1746–1828)
Self-portrait with Doctor Arrieta (1820)
Oil on Canvas (114.62 x 99.38 x 9.53 cm)
The Minneapolis Institute of Arts, The Ethel Morrison Van Derlip Fund

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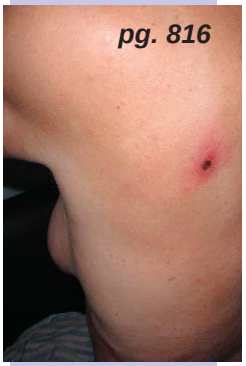
Research



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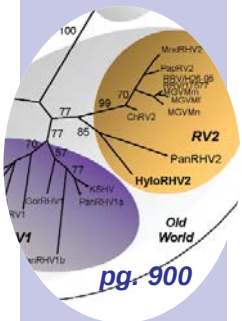

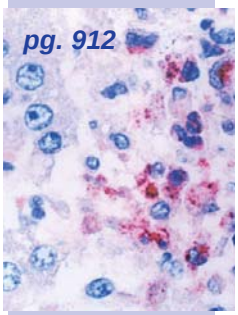

Segue

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News & Notes

Hospital Preparedness and SARS

Mona R. Loutfy,*† Tamara Wallington,‡ Tim Rutledge,† Barbara Mederski,† Keith Rose,† Sue Kwolek,† Donna McRitchie,† Azra Ali,† Bryan Wolff,† Diane White,† Edward Glassman,† Marianna Ofner,§ Don E. Low,¶ Lisa Berger,‡ Allison McGeer,¶ Tom Wong,§ David Baron,† and Glenn Berall†

On May 23, 2003, Toronto experienced the second phase of a severe acute respiratory syndrome (SARS) outbreak. Ninety cases were confirmed, and >620 potential cases were managed. More than 9,000 persons had contact with confirmed or potential case-patients; many required quarantine. The main hospital involved during the second outbreak was North York General Hospital. We review this hospital's response to, and management of, this outbreak, including such factors as building preparation and engineering, personnel, departmental workload, policies and documentation, infection control, personal protective equipment, training and education, public health, management and administration, follow-up of SARS patients, and psychological and psychosocial management and research. We also make recommendations for other institutions to prepare for future outbreaks, regardless of their origin.

On March 5, 2003, the first patient with severe acute respiratory syndrome (SARS) died in Toronto, Ontario, Canada. This index patient was a 78-year-old woman who, upon returning to Toronto from Hong Kong, transmitted the new variant coronavirus to her family (1). On March 7, her son was admitted to the hospital, and he subsequently died on March 13. His unrecognized disease led to nosocomial transmission of this disease in Toronto (1). As of August 28, 2003, a total of 375 cases of suspected and probable SARS had been identified in Toronto; most of these cases occurred within healthcare facilities (1–3). A minority of cases were related to household and community transmission, most acquired after hospital visits. The last community-acquired case of SARS-associated coronavirus (SARS-CoV) infection was identified on April 13, 2003 (2).

On the basis of the absence of new cases two incubation periods after the last case, barrier precautions were downgraded in Toronto hospitals on May 8, 2003. However, on May 23, the medical community realized that nosocomial transmission of SARS to patients and visitors had been occurring on a single ward in North York General Hospital (NYGH) throughout April and early May (3). Staff had been protected by personal protective equipment and therefore, because of the absence of staff cases and an epidemiologic link, the identification of the cases was delayed.

On May 23, a second phase of the outbreak (SARS II) was declared at NYGH, and the hospital was designated as a level-3 institution, which indicated that SARS had been transmitted through unprotected exposure (3). Consequently, a 10-day work quarantine for all staff was imposed. While this action prevented a major staffing shortage, it required all staff to wear N95 respirators at all times in the facility. When not at work, staff were at home, in home quarantine. During SARS II at NYGH, 55 patients were admitted with a diagnosis of SARS, and another 200 patients were assessed in the emergency department. We discuss the multidisciplinary and cross-departmental response used to establish SARS care at NYGH and offer recommendations that may help other hospitals prepare for an outbreak of SARS or any other infectious agent.

Building Preparation and Engineering

Wards

At the peak of SARS II, NYGH had 46 patients with investigated, suspected, or probable SARS in respiratory isolation in private, negative-pressure rooms (4). This was accomplished because two nearly constructed, empty, hospital wings were available. Within 72 hours of the declared outbreak, two units were converted into SARS wards, one with 22 rooms, the other with 27. Each private, negative-pressure room had no drapes and contained minimal equipment: one chair, a bedside table, a hamper for discarded linen, a garbage bin for contaminated equipment, and a hand sanitizer. Outside each room, a table held the personal protective equipment for staff entering the rooms. Outside each SARS ward were change-rooms for staff to change in and out of scrubs at the beginning and end of each shift.

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Intensive Care Unit

We learned that the intensive care unit (ICU)'s capacity is one of the factors that governs the number of SARS patients a hospital can manage. Since approximately 20% of patients with SARS require ICU care, the maximum number of patients with SARS that a hospital can manage can be calculated (5). At NYGH, the ICU's capacity was 22 rooms, which allowed the care of approximately 80 SARS patients in the hospital at any time. The ICU had private, self-contained, glass-enclosed rooms. The adjacent ward was a clean unit containing the standard ICU equipment as well as tables with personal protective equipment. Outside the ICU, a change-room was stocked with fresh scrubs and linen-disposal bins.

Emergency Department and SARS Assessment Clinic

Similar principles were applied to the emergency department, which had eight private, negative-pressure rooms. This department was closed to the public because of the hospital's level-3 status but stayed open for hospital employees and recently discharged patients. At the request of the provincial government, a SARS clinic was established to assess members of the public with symptoms of SARS. This clinic was constructed within 1 week in the 1,782–square foot ambulance bay. It contained eight negative-pressure isolation rooms built with pipe framing and plastic walls and ceilings. Areas for clerical work, registration, and changing personal protective equipment were also created. Other components included an area for case review, a lead-lined x-ray room, and an x-ray viewing room. A 40–x 20–foot tent was placed at the entrance of the clinic to provide ample space for a waiting area (Figure 1).

Figure 1. Tent assessment clinic built on ambulance loading dock for assessment of the general public for any symptom suggestive of severe acute respiratory syndrome. A, 40- x 20-foot tent constructed on the ambulance bay of the emergency department provided a spacious waiting area adjacent to the clinic area; B, inside the tent, eight cubicles were constructed with metal pipe frames and thick plastic walls, each ventilated with a custom-built ventilation system.

Engineering and Maintenance

The above-mentioned wards were considered SARS units, and the same engineering principles were applied to each. Each patient's room met the minimum requirement of six air changes per hour. Twice daily, the engineering department tested the negative-pressure status in all SARS units and patient rooms and presented the results to the hospital administration. In addition, an external company conducted daily assessments of the air circulation within the rooms. Highly trained engineering staff and clear blueprints and plans of the facility's ventilation system were needed to implement all the required changes.

We recommend that hospitals take the following building preparation and engineering steps to prepare for an emerging infectious disease: 1) complete an assessment of their current facilities and capabilities; 2) ensure that current blueprints of the facility's ventilation system are always accessible to facilitate expedient changes; 3) work with all relevant departments, including the proposed wards, ICU, and emergency department, to develop a strategy that allows for the rapid construction or conversion of the maximum number of private, negative-pressure rooms; and 4) identify in advance a timeline and areas of responsibility for constructing the maximum number of private rooms.

Personnel

Our biggest challenge during the outbreak was insufficient personnel. Most personnel were required at the beginning of each phase and were then needed for approximately 3-1/2 weeks. Although more personnel were recruited, they did not start work for 1 to 2 weeks after the initial influx of patients. We required additional nurses, unit managers, infection control personnel, housekeeping staff, ward clerks, and supply stocking and inventory staff.

Physicians recruited to manage the outbreak included primary-care doctors, infectious diseases consultants, hospital epidemiologists, public health physicians, emergency department physicians, and radiologists.

Nursing Staff

On the SARS wards, we aimed for a high ratio of nurses to patients. At the beginning of the outbreak, the ratio was approximately 4–5 patients per nurse, a potentially dangerous ratio that could lead to transmission. During SARS II, the ratio was 1:1 if the patient was on oxygen requiring hourly monitoring and 2:1 for more stable patients. In the ICU, the ratio was two nurses per patient, which allowed for one nurse in the room and another outside. To avoid transmission, nurses were extensively trained in SARS patient care, the use of personal protective equipment, the potential risks for transmission, and preparedness for high levels of stress.

Housekeeping

Dedicated and well-trained housekeepers were very important during the outbreak. Our housekeepers were trained in proper cleaning techniques and the use of personal protective equipment.

Physicians

At NYGH, we used a most responsible physician (MRP) model for patient care, i.e., a primary care doctor (including emergency department physicians, general internists, family physicians, surgeons, and anesthesiologists who volunteered to care for SARS patients) directly cared for the patients. The patient-to-physician ratio for the MRP was 5–10 SARS patients per physician. One infectious disease consultant was assigned to each SARS ward, and one also covered the SARS ICU for a ratio of 20 to 30 SARS patients per infectious disease consultant. The MRP conducted all direct patient care, reviewed all cases, wrote the notes and, at mid-day, reviewed all cases with the infectious disease consultant, who was also responsible for alerting the MRP of new developments pertinent to SARS, for making changes in patient management, consulting with the emergency department for SARS assessments, and communicating with the onsite public health physician and outbreak management team. Training the emergency department physicians in SARS procedures was vital; our emergency department physicians became adept at evaluating potential SARS cases, which resulted in fewer patients being referred to infectious disease consultants.

We recommend that hospitals take the following personnel steps in advance of an emerging infectious disease event: 1) Calculate the maximum number of beds available for conversion to negative-pressure rooms on the wards, in the ICU, and the emergency department. The resulting figure will indicate the number of staff (including nurses, allied healthcare workers, and physicians) required from day 1. 2) Develop a system to identify those staff members who would be available to start working as part of the outbreak team within 24 hours. Such staff must be prepared for training and able to commit their services for a minimum of 3 to 4 weeks. 3) Generate a plan to meet the extra cost of hiring vital personnel (the greatest economic cost during such an outbreak). 4) Prepare for intensive training of both skilled staff and all other hospital employees in the use of personal protective equipment and infection-control procedures.

Departmental Work Load

The SARS outbreak affected every hospital department. After NYGH was identified as a level-3 institution, only the two SARS units, the SARS ICU and the emergency department, continued to function. Most non-SARS patients were discharged, which left only 20 patients in this 400-bed hospital. However, every department's continued contribution was needed. Occupational health played a major role in reviewing which healthcare workers could return to work. Environmental services and housekeeping were greatly affected by additional requirements throughout the hospital. Security ensured that unauthorized persons did not enter the hospital; a security staff member, with a nurse, escorted SARS patients on transports between departments, logging the date, time, and persons involved in the transfer. Because of the isolation measures, SARS patients' x-rays were taken with portable machines; two technicians were needed. The laboratory was overloaded due to the increased number of daily samples, which required a blood technician system to collect all samples at 7:00 a.m. Pharmacy staff handled increased orders and organized Health Canada's approval requirements for ribavirin and interferon.

We recommend that hospitals conduct a review of each department's existing capacity and capabilities for handling an outbreak. Strategies should then be developed to address any deficiencies.

Policies, Procedures, and Documentation

During the outbreak, Toronto hospitals developed standardized systems for all implicated procedures, including code blues, patient transfers, and other infection-control procedures.

Of vital importance was the policy for patient oxygenation and early transfer to the ICU. SARS patients in need of oxygen can deteriorate rapidly, requiring intubations within 6 to 12 hours, a high-risk procedure that can lead to further nosocomial transmission (6). At NYGH, patients who had an oxygen saturation $\leq$92% and needed any

amount of supplemental oxygen had their vital signs with oxygen saturation monitored every 2 hours instead of every 4 (6). If patients required more than 4 L/min of oxygen, the monitoring increased to every hour. Once patients required >6 L/min of oxygen, they were transferred to the ICU. Such early transfer allowed for elective, early intubation to be done in a controlled environment by minimal staff, which resulted in a reduced risk for transmission. Staff at intubations wore T4 Personal Protection Systems (Stryker Instruments, Kalamazoo, MI), although these items were not proven to be beneficial (6).

Specific standardized forms were developed, including emergency department SARS consult sheets that included all the appropriate key questions regarding exposure, date of onset of symptoms, specific symptoms, laboratory investigations, and chest x-ray findings; admission order forms, which allowed for standard orders for the nurses and MRPs (see Appendix, online only; available from: www.cdc.gov/ncidod/eid/vol10no5/03-0717_app.htm); and progress note forms, which documented symptoms, temperature, oxygen saturation and requirement, laboratory and x-ray results, and the daily plan. These documents both streamlined the process of daily review of 10 to 20 patients and standardized the level of care.

We recommend that hospitals do the following: 1) consider obtaining existing documentation and policies from other hospitals, such as NYGH; 2) develop an organized process of documentation that will facilitate an organized response to patient needs; and 3) assess different systems equivalent to the T4 Personal Protection Systems (Stryker Instruments) for particle removal efficiency and air-flow rate to choose the optimal system before an outbreak.

Infection-Control Service

Before the SARS outbreak, NYGH had only two infection-control practitioners (ICPs). During the outbreak, additional ICPs were recruited, and hospital epidemiologists from other institutions arrived to create a system and infrastructure for infection control. We expanded an extant infection-control team to organize all the policies, systems, and structures for future infection control. Extra staff included a coordinator, four ICPs, a nurse clinician, a public health nurse, an administrator, a hospital epidemiologist (an infectious disease specialist with training in hospital epidemiology), and a clinical infectious disease physician. Members of this team made daily ward rounds to answer questions and conduct surveillance for fever and symptoms. In addition, they met several times a week to review policies, coordinate teaching, and address all other issues. To ensure consistent levels of infection-control practice, a system that reviewed the quality of practice was established: it was essential for the ICPs to maintain some degree of authority on these issues.

Because infection-control issues are vitally important, a hospital should do the following: 1) identify an appropriate number of ICPs for the hospital size (7); 2) include a qualified hospital epidemiologist on the infection-control team; 3) include a public health physician or designate on the team; 4) maintain constant vigilance during symptom surveillance; 5) sustain excellent standards of staff training and communication and apply continuous monitoring of infection-control practices; 6) ensure that all policies and documentation go through this team; and 7) provide the team with the necessary authority to work effectively throughout the hospital.

Personal Protective Equipment and Fit-Testing of Respirators

The constant availability and use of personal protective equipment (much of which was disposable) was essential during the outbreak, including the following: N95 respirators, goggles, face shields, hair nets, gowns, and scrub suits. Specific policies and procedures were developed for putting on and removing personal protective equipment. For example, before entering a SARS patient's room, a staff member wore an N95 respirator, goggles, face shield, hair net, a gown over scrubs, and two pairs of gloves. The order in which personal protective equipment was removed when a staff member exited a patient's room was exact. For example, inside the room by the door, the first pair of gloves was removed, followed by the hair net, the face shield, and the second pair of gloves; next, hands were washed with quick-drying antiseptic solution, and the gown was carefully removed; then the hands were washed again before the staff member left the room. In the hallway, hands were washed, goggles removed and disposed of, hands washed again, respirators removed, hands washed, and finally, a new N95 respirator was donned. During SARS II, the provincial government issued a directive requiring that respirators be fit-tested at all hospitals. This recommendation proved to be difficult to implement at NYGH because we were in the midst of an outbreak.

We recommend that, in advance of an outbreak, hospitals should do the following: 1) prepare clear policies on the proper use of personal protective equipment during such an outbreak; 2) ensure that adequate supplies of essential items required for personal protective equipment will be available or are already stocked; 3) have staff fit-tested for respirators and document results or obtain forms of higher level respiratory protection that do not require fit-testing (e.g., loose-fitting powered air-purifying respirators).

Education and Training

All staff had to be trained and educated on every aspect of SARS, including the proper use of personal protective

equipment, risks to themselves and their families, and infection-control policies and procedures. At NYGH, training was conducted by a group of nurse clinicians assigned to each unit. Daily full-day, mandatory training sessions for all staff working on the SARS wards were created and included topics such as the proper way to don personal protective equipment, the psychological impact of SARS, and general infection-control practices.

We recommend that hospitals do the following: 1) include all departments in training, preferably in advance of an outbreak; 2) develop a program for certification in "Readiness to Manage an Infectious Medical Disaster Outbreak"; 3) develop a "train-the-trainer" model together with continued quality assurance monitoring.

Public Health Outbreak Management Team

One unique feature of this outbreak was the formation of a mobile public health outbreak management team. It included two physicians, a manager, and five investigators (either public health nurses or inspectors), who were stationed beside the hospital coordinators and infectious disease specialists and remained onsite 24 hours each day for 4 weeks. This setup promoted outstanding communication and excellent relations between all parties, which allowed rapid exchanges of information that led to swift contact tracing and the quarantine of persons identified as having had unprotected exposure to a SARS patient. The public health nurses attended morning ward meetings to review management plans for patients admitted overnight, followed patient progress directly on the wards, and attended the regular infection-control team meetings.

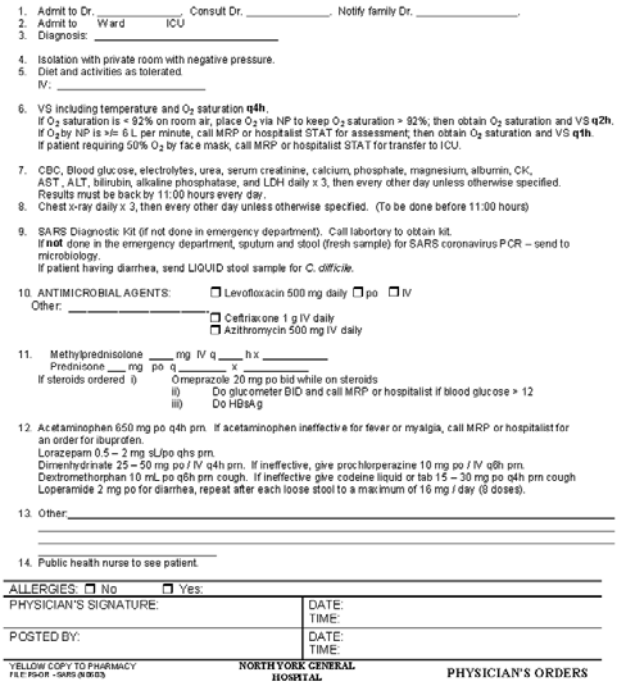
We recommend that the healthcare system do the following during an outbreak: 1) facilitate effective communication between public health and hospital staff; and 2) establish a common information technology platform that allows for a streamlined, accessible flow of data between jurisdictions.

Management and Administration

At NYGH, a 24-hour command center administered all the details connected to managing the outbreak and answered all questions. Department heads met daily at 9:00 a.m., which allowed them to impart important information to their staff. At 11:00 a.m., the SARS management committee held a meeting at which all decisions for the hospital were subsequently implemented. The key front-line players—including unit managers from each ward, infection control, infectious disease, and the chief of medicine—met daily to exchange information and properly manage the outbreak. Forums were regularly held by the hospital president to answer questions from the staff. All media contacts went through the single public relations department, to transmit a single message during this controversial time. We recommend that hospital administrations be prepared to play a pivotal role during such an outbreak.

SARS Follow-up Clinic

Patients recovering from SARS were discharged to remain in quarantine at home for an additional 7–10 days. Follow-up appointments were made for days 11 and 30 postdischarge. A single physician coordinated and ran the SARS follow-up clinic out of the emergency department. Again, a list of standard, step-by-step procedures was made for the assessment, and a checklist was designed (Figure 2). The physician assessed symptoms and reviewed follow-up laboratory tests. Convalescent-phase serologic tests were conducted 3–4 weeks after the onset of symptoms, and if results of a polymerase chain reaction test were positive for SARS-CoV, the test was repeated. Follow-up chest x-rays and, occasionally, computed tomographic scans were performed. A psychologist and a social worker provided psychological assessment and support during the follow-up visits. For SARS follow-up clinics in other hospitals, we recommend that, using standardized and organized methods, the hospitals prepare plans for an extensive follow-up system.



1. Admit to Dr. _____. Consult Dr. _____. Notify family Dr. _____.
2. Admit to Ward ICU
3. Diagnosis: _____
4. Isolation with private room with negative pressure.
5. Diet and activities as tolerated.
   IV: _____
6. VS including temperature and O_2 saturation **q4h**.
   If O_2 saturation is < 92% on room air, place O_2 via NP to keep O_2 saturation > 92%; then obtain O_2 saturation and VS **q2h**.
   If O_2 by NP is >= 6 L per minute, call MRP or hospitalist STAT for assessment; then obtain O_2 saturation and VS **q1h**.
   If patient requiring 50% O_2 by face mask, call MRP or hospitalist STAT for transfer to ICU.
7. CBC, Blood glucose, electrolytes, urea, serum creatinine, calcium, phosphate, magnesium, albumin, CK, AST, ALT, bilirubin, alkaline phosphatase, and LDH daily x 3, then every other day unless otherwise specified.
   Results must be back by 11:00 hours every day.
8. Chest x-ray daily x 3, then every other day unless otherwise specified. (To be done before 11:00 hours)
9. SARS Diagnostic Kit (if not done in emergency department). Call labortory to obtain kit.
   If **not** done in the emergency department, sputum and stool (fresh sample) for SARS coronavirus PCR – send to microbiology.
   If patient having diarrhea, send LIQUID stool sample for *C. difficile*.
10. ANTIMICROBIAL AGENTS: ❒ Levofloxacin 500 mg daily ❒ po ❒ IV
    Other: _____
    ❒ Ceftriaxone 1 g IV daily
    ❒ Azithromycin 500 mg IV daily
11. Methylprednisolone ____ mg IV q ____ h x _____
    Prednisone ___ mg po q ____ x _____
    If steroids ordered i) Omeprazole 20 mg po bid while on steroids
    ii) Do glucometer BID and call MRP or hospitalist if blood glucose > 12
    iii) Do HBsAg
12. Acetaminophen 650 mg po q4h prn. If acetaminophen ineffective for fever or myalgia, call MRP or hospitalist for an order for ibuprofen.
    Lorazepam 0.5 – 2 mg sL/po qhs prn.
    Dimenhydrinate 25 – 50 mg po / IV q4h prn. If ineffective, give prochlorperazine 10 mg po / IV q6h prn.
    Dextromethorphan 10 mL po q6h prn cough. If ineffective give codeine liquid or tab 15 – 30 mg po q4h prn cough
    Loperamide 2 mg po for diarrhea, repeat after each loose stool to a maximum of 16 mg / day (8 doses).
13. Other: _____
14. Public health nurse to see patient.

ALLERGIES: ❒ No ❒ Yes:

PHYSICIAN'S SIGNATURE:	DATE: TIME:
POSTED BY:	DATE: TIME:

YELLOW COPY TO PHARMACY FILE FSOR - SARS (9/03/03) — NORTH YORK GENERAL HOSPITAL — PHYSICIAN'S ORDERS

Figure 2. Standardized follow-up checklist of patients with severe acute respiratory syndrome. BP, blood pressure; CBC, complete blood count; LDH, lactate dehydrogenase; AST, aspartate transaminases; PCR, polymerase chain reaction; RR, respiratory rate; P, pulse; ALP, alkaline phosphatase; CK, creatine kinase; ESR, erythrocyte sedimentation rate; Ca, calcium; PO^4, phosphate; Mg, magnesium; F/u, follow-up; NP, nasopharyngeal; PA, posterior-anterior.

Psychological and Psychosocial Management

Psychological and psychosocial support for both patients and the entire hospital staff was necessary during the SARS outbreak. Staff were affected by the fear of contracting and transmitting this new disease; SARS patients experienced stress because of their isolation, fear for their lives, guilt, anger, anxiety, and depression.

At NYGH, we put together a SARS psychological team (including social workers, psychiatric crisis nurses, psychiatrists, and infectious disease specialists) that developed a plan to manage the psychological impact on patients and staff. Patients were seen at least twice weekly. A social worker phoned each patient on days 2 and 6 postdischarge to follow up. A psychiatric crisis-line phone number was given to every patient in case he or she needed urgent attention. An outpatient system with psychiatrists was put in place to handle posttraumatic stress syndrome. These services were also established for all hospital staff. A quiet staff room was available for relaxation or discussion with a team member. After the outbreak, debriefing sessions were held with trained psychologists and counselors. We recommend that hospitals' psychiatry departments, in conjunction with their hospital administration, develop a response plan for a crisis outbreak.

Research

Research is imperative during such an outbreak, particularly for a new disease. The physicians and staff who were managing the outbreak had minimal time to do research, but they had many urgent questions. At NYGH, infectious disease and internal medicine physicians from Health Canada, Toronto Public Health, and other organizations came in to help with the research. The ethics board was prompt in attending to required approvals, often a lengthy process. Funding for such emergency research—another important factor—was provided.

To facilitate emergency research, we recommend that hospitals do the following: 1) identify potential members for collaborative research before an outbreak; 2) establish an expedient process for ethics approval; and 3) be prepared to alert funding agencies for the need for additional funding and support.

Conclusion

A multidisciplinary approach to manage the second phase of the SARS outbreak in Toronto was undertaken at NYGH. This successful approach was only possible with the hard work and collaboration of many people as well as open and active communication maintained among all departments, employees, and patients. Many lessons, taken from this experience, can be applied by hospitals preparing themselves for such an outbreak. Finally, the policies, procedures, and documents developed at our institution and others are freely available to other centers to review and adapt as appropriate.

Acknowledgments

We thank all staff members of North York General Hospital (NYGH), Toronto Public Health, Ontario Ministry of Health and Long-term Care, and Health Canada, whose endless hours helped to manage the outbreak; Paula Chabanais and Deirdre MacLean for expert editing of the manuscript; and NGYH nurse clinicians for providing training, infection control team members for developing policies, intensive-care unit staff for their dedication to patient care, and social workers for their psychological support.

Dr. Loutfy is a Canadian Institutes of Health Research postdoctoral fellow at McGill University, Montreal, Quebec. She specializes in clinical HIV research. She was responsible for the care of more than 75 SARS patients in Toronto.

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SARS in Healthcare Facilities, Toronto and Taiwan

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The healthcare setting was important in the early spread of severe acute respiratory syndrome (SARS) in both Toronto and Taiwan. Healthcare workers, patients, and visitors were at increased risk for infection. Nonetheless, the ability of individual SARS patients to transmit disease was quite variable. Unrecognized SARS case-patients were a primary source of transmission, and early detection and intervention were important to limit spread. Strict adherence to infection control precautions was essential in containing outbreaks. In addition, grouping patients into cohorts and limiting access to SARS patients minimized exposure opportunities. Given the difficulty in implementing several of these measures, control measures were frequently adapted to the acuity of SARS care and level of transmission within facilities. Although these conclusions are based only on a retrospective analysis of events, applying the experiences of Toronto and Taiwan to SARS preparedness planning efforts will likely minimize future transmission within healthcare facilities.

In March 2003, reports of healthcare workers with unexplained pneumonia in Vietnam initiated an international investigation of the infection that came to be known as severe acute respiratory syndrome (SARS) (1). The cause of SARS was later identified as a coronavirus, which was cultured from specimens provided by a healthcare worker who subsequently died of SARS (2). During the outbreak, transmission in hospitals and infection in healthcare workers persisted. In Toronto and Taiwan, nosocomial transmission played a substantial role in initiating and maintaining outbreaks of SARS. We summarize our experiences during these outbreaks to highlight key factors that can help healthcare and public health officials prevent nosocomial transmission of SARS. In addition, we offer conclusions based on an in-depth, retrospective analysis of the events as they unfolded in these two settings.

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High Risk for Transmission in Healthcare Workers, Patients, and Visitors

At the onset of the global outbreak, patients infected with SARS coronavirus (SARS-CoV) sought care at emergency departments for symptoms of what appeared to be common respiratory infections. During such encounters, minimal infection control measures were implemented since most known infections did not warrant them. However, in some circumstances, conditions were favorable for efficient transmission of SARS. Many exposed healthcare workers, patients, and visitors became infected and subsequently transmitted infection to others in their healthcare facilities. Nosocomial transmission was the primary accelerator of SARS infections, accounting for 72% of cases in Toronto (3) and 55% of probable cases in Taiwan (Table) (4).

In Toronto, the outbreak unfolded in two phases, both attributable to nosocomial transmission (Figure 1). The first phase resulted from a case of unrecognized SARS in an infected contact of a recent traveler to Hong Kong (5). The second phase resulted from unknown transmission of SARS among hospitalized patients during a period when healthcare workers were being instructed to wear personal protective equipment, including gowns, gloves, and masks (6). In Taiwan, the outbreak had two phases (Figure 1). The first phase consisted of sporadic SARS cases in travelers without nosocomial transmission (7). In the second phase, transmission at one municipal hospital ignited a number of subsequent nosocomial outbreaks when SARS patients were transferred to other facilities (4).

A number of factors may make nosocomial transmission a common mode of infection. Unlike many other viral respiratory diseases in which the concentration of virus is greatest on disease onset, the concentration of SARS-CoV in secretions appears to peak approximately 10 days after symptom onset (8) when a patient's symptoms are often worsening and may require medical attention. Thus, patients may be most capable of transmitting the virus at the point when they encounter healthcare workers. In addition, transmission appears to be primarily through exposure to respiratory droplets and direct contact with patients

Table. Characteristics of the SARS outbreak in the greater Toronto area and Taiwan, March–June 2003[a]

Characteristic	GTA, no. (%)	Taiwan, no. (%)[b]
Total cases	375	NA
Probable	247 (66)	668
Suspected	128 (34)	NA
Deaths	44 (12)	72 (11)
Healthcare related	271 (72)	370 (55)
Healthcare workers	164 (44)	120 (18)
Patients or visitors	107 (28)	256 (38)
Hospitals with hospitalized SARS patients	23	84
Hospitals with SARS transmission	10 (43)	8 (10)
Hospitals that closed wards or an emergency room	10 (43)	NA

[a]SARS, severe acute respiratory syndrome; GTA, greater Toronto area; NA, data not available
[b]Percentage expresses proportion of all probable SARS cases

and their contaminated environment (5,9). Healthcare workers and others in contact with SARS patients may be more likely to become infected, especially if exposed during aerosol-generating procedures (i.e., intubation, nebulizing medications). Finally, even after recognition of SARS, lapses in infection control measures may be responsible for infection in healthcare workers.

Whether SARS will occur again, and if so, whether the epidemiology will be similar to the outbreak in the spring of 2003 are not known. However, given the severity of illness seen in SARS patients and their eventual need for medical attention, healthcare workers and others in healthcare facilities are likely to remain at high risk if SARS reemerges.

Variation in SARS Communicability

Over the course of the SARS outbreak, certain persons and settings were found to be more efficient at transmitting SARS-CoV infection than others. In Taiwan, after an initial period of apparent control of SARS by public health officials (7), exposures to an apparent "super-spreader" with SARS contributed to an explosion of infections at a municipal hospital in Taipei (4). An infected hospital laundry attendant continued working despite worsening symptoms of diarrhea and pneumonia. Between the onset of his illness and eventual recognition of SARS, exposures to the worker and to the hospital led to at least 137 probable cases, including 45 in healthcare workers. Similarly, a small number of persons also generated a large number of cases during the first phase of the Toronto outbreak when a cluster of healthcare workers were infected with SARS after the intubation of a severely ill SARS patient (10). Comparable transmission from one person to many was seen in Singapore as well (11).

In contrast, experiences with SARS in the United States and several other countries have not shown similar super-spreading patients or events despite opportunities for transmission (12). The reasons for such variable communicability are uncertain but may be due to innate characteristics of infected patients (13), high virus concentrations in secretions during peak illness (8), or exposures to aerosol-generating procedures such as intubation or positive-pressure ventilation (10). Because these procedures are considered high risks for SARS transmission, guidelines were developed that emphasize use of PPE and, if needed, furlough for healthcare workers with unprotected exposure to these procedures (14).

Transmission from Unrecognized Cases

On February 23, 2003, a 78-year-old Canadian woman returned from a visit to Hong Kong. While there, she had unknowingly been infected with SARS-CoV during her stay at a hotel in Kowloon (5). After returning to Toronto, the patient's condition worsened, and she died at home. SARS developed in her son, and he was hospitalized with respiratory distress on March 7. Before his death on March 13, he infected two other patients and one healthcare worker, all of whom subsequently exposed others to the infection before SARS was eventually recognized. Infected visitors also contributed to transmission in the hospital. Ultimately, 128 cases were associated with this hospital outbreak, including 47 (37%) hospital staff and 36 (28%) patients and visitors (5). Many of these cases occurred early in the global outbreak and before SARS transmission was recognized in Canada. Once the disease was recognized, appropriate infection control practices were initiated so that by May 14, the World Health Organization advised that Toronto was no longer an "affected area" with the last locally acquired, recognized case having occurred on April 20, 2003.

After the first phase of SARS in Toronto, healthcare workers continued to use extensive personal protective equipment (e.g., routine contact precautions with an N95

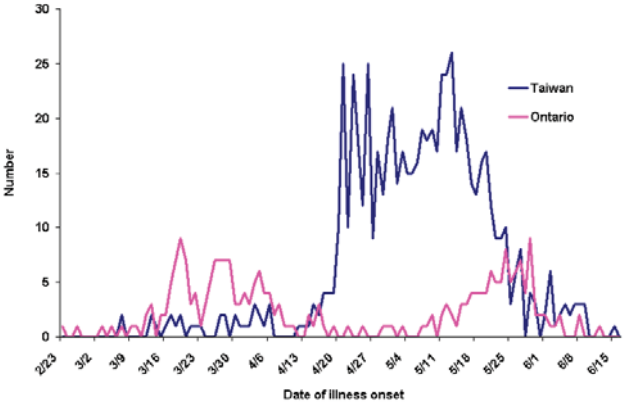

Figure 1. Number of probable cases of severe acute respiratory syndrome, by location and date of illness onset—Toronto and Taiwan, February 23–June 15, 2003.

or equivalent respirator). However, after a period with no apparent SARS transmission, public health officials relaxed the requirement for extensive PPE. Subsequently, a cluster of SARS cases occurred among healthcare workers, followed by the second phase of SARS transmission in Toronto (6). In retrospect, investigators determined that SARS-CoV transmission had continued undetected among patients. These unrecognized cases occurred later in the global outbreak and after recognition of SARS transmission in Canada.

The experiences from Taiwan and from both outbreak phases in Toronto underscore the difficulty in detecting SARS cases and the cascade of infections that can occur from even one unrecognized case among persons in the hospital. Symptoms of SARS are nonspecific and may represent infection due to a number of respiratory pathogens. Without rapid diagnostic tests, clinicians must rely for diagnosis on a patient's history of travel, exposure to healthcare facilities, or contact with patients with suspicious cases of pneumonia. To prevent SARS transmission, all healthcare workers and visitors entering hospitals in Toronto and Taiwan were screened for symptoms or epidemiologic links to settings where transmission was known or suspected. Epidemiologic links are important discriminators for considering a diagnosis of SARS; however, before any global SARS activity and during periods of notable local transmission, these epidemiologic links may lose their discriminating ability. Ultimately, vigilant and intuitive clinicians may be the best means of recognizing cases of SARS.

Minimizing Transmission through Early Detection and Intervention

Hospital emergency departments were important sites for SARS transmission during the early part of the outbreak in Toronto (5). In Taiwan, transmission in the emergency department occurred through unrecognized case-patients and during a period when infection control measures were weakened due to the rapid influx of SARS patients seeking evaluation. A number of administrative, engineering, and other controls were eventually implemented to minimize transmission of SARS in emergency departments in both Toronto and Taiwan. One important activity was "triage screening." For this, a questionnaire was administered to entrants to identify SARS symptoms and exposures. Screening was accompanied by a temperature check, mandatory hand hygiene by the patient, and often by providing a surgical mask before admission to the hospital. These precautions were taken when the patient was first encountered by hospital staff.

At the peak of the outbreaks in Toronto and Taiwan, healthcare providers and public health officials were faced with the possibility that any person coming to an emergency department with a febrile respiratory illness might have SARS and might transmit infection to other patients. In response, officials either constructed or retrofitted existing facilities to create SARS evaluation centers (i.e., "Fever Clinics") (13). These units were designed to safely assess large numbers of people while minimizing the risk for SARS transmission, and in fact in both Toronto and Taiwan, no transmission was reported in these facilities. Staff and patients were grouped into cohorts, and a space of >l m was allocated between patients to make direct contact and droplet transmission less likely. Dedicated entrances and exits and clearly marked patient pathways were provided to segregate patients under evaluation. Provisions were made to ensure adequate ventilation and air exhaust to reduce the risk for droplet or airborne transmission. In Taiwan, temporary structures with high efficiency filtration were built (Figure 2). In Toronto, both tents and existing facilities were used.

Figure 2. A, evaluation center for severe acute respiratory syndrome (SARS) in Toronto, demonstrating spatial separation of chairs in waiting area intended to reduce patient-to-patient transmission. B, evaluation center for SARS in Taiwan, demonstrating triage screening of a patient by a healthcare worker wearing personal protective equipment.

Strict Adherence to Infection Control Practices

Early in the global outbreak, SARS-CoV was frequently transmitted to healthcare workers. At that time, with no diagnostic assays or therapies, public health officials recommended personal protective equipment to prevent contact, droplet, and airborne transmission (14). In this situation, a large number of healthcare workers were required to wear gowns, gloves, N95 or higher respirators, and eye protection, often for hours. In the past, this level of protection had been recommended infrequently for those treating patients with such infections as active multidrug-resistant tuberculosis, suspected smallpox, or viral hemorrhagic fever (15). In Toronto and Taiwan, nurses, physicians, and housekeeping and other ancillary staff required rapid training to familiarize them with appropriate technique for the use of PPE. Additionally in Taiwan, training was needed for family and hired caretakers who provided a supportive healthcare function in hospitals in Taiwan (4).

To facilitate the complicated process of donning and removing personal protective equipment, officials developed videos, computer presentations, and posters to train and remind healthcare workers. In addition, changes in shift duration and rearrangement of nursing stations in SARS wards were needed to minimize lapses in infection control. However, as mentioned, even with fully protected healthcare workers, SARS transmission continued among patients, precipitating the second outbreak phase in Toronto. Glove use outside the immediate care environment was not recommended, but officials needed to emphasize the importance of removing gloves and washing hands after leaving SARS areas to prevent contaminating the environment or infecting patients.

Experiences in Toronto (5), Taiwan (4), and globally (9) indicate that the primary mode of SARS transmission is through direct contact and respiratory droplets. However, the cluster of SARS cases in Toronto healthcare workers after the intubation of a patient (10), as well as other reported superspreader events, suggest the possibility of limited airborne transmission under certain circumstances. Hand hygiene, one of the most important and simplest of interventions, was widely advocated both in the hospital and in public places. Surgical masks and respirators were recommended equipment for healthcare workers; however, use of masks and respirators in Taiwan became commonplace both in and outside the hospital. Inappropriate use of PPE caused shortages of supplies. In response, officials developed guidelines for respirator reuse and identified alternatives for equipment in short supply.

Infection control in Toronto and Taiwan became an essential public health activity, which required the implementation of precautions beyond most officials' experience and expectations. Public health authorities took an active role in assessing the adequacy of control measures in hospitals and in investigating any potential transmission. Once widespread infection-control practices, along with other measures, were implemented, the number of new SARS cases declined.

Minimizing Exposure Opportunities through Patient Isolation

Instituting recommended airborne transmission precautions for SARS patients required the use of airborne-infection isolation rooms, also known as "negative pressure" rooms. During early control of SARS in Taiwan, the small number of imported cases was adequately contained in these isolation rooms (7). After the rapid increase of cases, affected hospitals quickly exceeded their capacity to accommodate all patients in such isolation rooms. Two initiatives addressed the problem. First, government officials provided resources to build new airborn-infection isolation rooms at hospitals (4). Second, hospital officials grouped SARS patients in private rooms on dedicated, reengineered, SARS wards with modified ventilation systems that separated the ward airspace from the remainder of the hospital. Barriers of plastic sheeting and tape were constructed to limit access. When possible, SARS patients with pneumonia, who presented the highest risk for transmission, were placed in airborne-infection isolation rooms; other SARS patients were placed in private rooms on the SARS wards. Restricting SARS care to one unit or ward allowed the separation of contagious and noncontagious patients and limited the number of staff with potential exposures to SARS. Exposure opportunities were further minimized by maintaining a high staff-to-patient ratio and a high level of infection-control training on SARS wards.

In both Toronto and Taiwan, hospital officials restricted access to affected hospitals by limiting the number of entryways. Access stations were staffed with personnel to screen for fever, symptoms, or potential SARS exposures. Few visitors to SARS patients were allowed, and healthcare workers or visitors exposed to facilities where SARS transmission had occurred were not permitted to enter non-SARS areas. Hospitals with notable recent nosocomial transmission prevented visitors or nonessential staff from entering. Measures to limit access also included restrictions for transferring patients into or out of the hospital. If medically necessary, transfers were made after consultation with hospital and public health authorities.

Officials in Toronto and Taiwan considered designating a single facility to serve as a "SARS hospital" for their jurisdictions. However, implementing this policy was challenging. Facilities that were not seriously affected generally did not want to become the principal providers of SARS care because of concerns regarding liability, impact on finances, and negative public image. Ultimately, public

health and healthcare officials chose to prepare and support many hospitals to provide care to SARS patients. This measure eliminated the need for a designated SARS hospital while maintaining a higher vigilance for SARS transmission at multiple facilities. In the second phase of the Toronto outbreak, four facilities where SARS patients were already residing were designated as SARS hospitals.

Adapting SARS Control Measures to a Facility

Many infection control activities in Toronto and Taiwan were resource intensive and difficult to maintain for an extended period. To prevent unnecessary use of staff and materials, some measures were implemented only when transmission in the surrounding community or within the hospital reached a particular level. For example, using surgical masks throughout a hospital to contain infection in a healthcare worker or other person with symptoms was only implemented when transmission in the community was ongoing or recent transmission had occurred in the facility. Other functions, such as limiting access, restricting transfers, and performing surveillance for new-onset illness among healthcare workers, were initiated at different times in hospitals on the basis of hospital transmission or community transmission.

Closing an emergency department or hospital ward also was linked to the level of transmission within a hospital. Closings were necessary to prevent additional cases in a hospital where the risk for transmission was high or the source of transmission was unknown. However, given the substantial negative effect on hospital finances and healthcare access in a community, the decision to close a hospital to new admissions was made only in consultation with public health authorities.

Conclusions

On July 5, 2003, the World Health Organization declared the world free of ongoing SARS transmission (16). However, the factors that led to the emergence of SARS are likely still in place, permitting the possibility that SARS will reemerge. If this happens, nosocomial transmission and cases among healthcare workers may also occur. Taking the experiences from Toronto and Taiwan and applying them to preparedness and prevention efforts likely will minimize SARS transmission in healthcare facilities.

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SARS in Hospital Emergency Room

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Thirty-one cases of severe acute respiratory syndrome (SARS) occurred after exposure in the emergency room at the National Taiwan University Hospital. The index patient was linked to an outbreak at a nearby municipal hospital. Three clusters were identified over a 3-week period. The first cluster (5 patients) and the second cluster (14 patients) occurred among patients, family members, and nursing aids. The third cluster (12 patients) occurred exclusively among healthcare workers. Six healthcare workers had close contact with SARS patients. Six others, with different working patterns, indicated that they did not have contact with a SARS patient. Environmental surveys found 9 of 119 samples of inanimate objects to be positive for SARS coronavirus RNA. These observations indicate that although transmission by direct contact with known SARS patients was responsible for most cases, environmental contamination with the SARS coronavirus may have lead to infection among healthcare workers without documented contact with known hospitalized SARS patients.

The coronavirus responsible for the severe acute respiratory syndrome (SARS-CoV) rapidly spread from Mainland China to 30 countries worldwide (1–4). From November 1, 2002, through July 31, 2003, a total of 8,098 probable cases were reported, including 346 from Taiwan (2). The disease is of great concern because of the high case-fatality rate, short incubation period, rapid spread along international air routes, and the large number of cases in previously healthy hospital staff (1,2,5–7). SARS appears to be spread most commonly by close person-to-person contact through exposure to infectious droplets and possibly by direct contact with infected body fluids (1,5–7). Emerging evidence indicates that SARS can be acquired from contaminated inanimate objects in the environment (8).

Taiwan is geographically close to China and Hong Kong and has a population of 23 million. An outbreak began on April 23, 2003, at a municipal hospital (hospital A) in Taipei. The index patient had unrecognized SARS. Multiple patients, visitors, and healthcare workers were exposed to this patient (9). After the outbreak at hospital A, patients sought care at the National Taiwan University Hospital, and patients with febrile illness screened in the emergency room (ER) increased substantially.

On May 8, 2003, we identified and reported to the local health department three SARS cases in patients whose only contact history was being treated at the National Taiwan University Hospital ER. Source and contact tracing failed to identify the index patient. In response to this outbreak, we admitted all ER patients in phases to a special unit where droplet and contact precautions were implemented, and on May 12, 2003, the operation of the ER was suspended.

On the same day, the infection control team was informed that three healthcare workers who worked in the ER had fever. They were immediately isolated, and initial interviews with the healthcare workers failed to identify a common source of infection. To better understand the mode of transmission, we conducted this epidemiologic study and environmental surveillance by using a highly sensitive and specific assay for SARS-CoV RNA. We describe how we traced the index patient to hospital A and the subsequent occurrence of three clusters of SARS after exposure to the National Taiwan University Hospital ER. We also provide evidence for indirect-contact transmission among some of the healthcare workers on the basis of the environmental studies.

Materials and Methods

Hospital Setting

The National Taiwan University Hospital is a 2,400-bed teaching hospital that provides both primary and tertiary care. National Taiwan University Hospital is located in

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downtown Taipei not far from hospital A. The ER is organized into several sections, including triage, examination, observation, critical care, and a clean area reserved for staff activities. A SARS screening unit was established on March 17, 2003, to interview and triage febrile patients with suspected cases of SARS. The patients were questioned about the presence of fever, myalgia, gastrointestinal or respiratory symptoms, whether they had close contact with a SARS patient, and recent travel. N95 respirators were placed on patients suspected to have SARS early during the triage process, and they were immediately placed in private rooms (SARS area) to avoid contact with others in the ER. A daily record was maintained of all patients screened in the SARS screening unit. SARS cases were defined according to the World Health Organization criteria (10), modified to expand the definition of contact to include any healthcare setting with nosocomial transmission.

Infection-Control Measures

Since March 14, 2003, infection-control measures required that all healthcare workers who had contact with patients with SARS use personal protective equipments, including gown, gloves, N95 respirators, disposable cap, and shoe covers. Later, a face shield was included for healthcare workers with close contact to SARS patients.

Healthcare workers who had any contact with SARS patients or their environment were placed under surveillance for 14 days after the last exposure. Those who had unprotected exposure, or those who were protected but had high-risk exposures to SARS patients were excluded from new duty assignments and were restricted from direct patient care and contact with other healthcare workers. Any healthcare worker in whom fever developed was placed in specially designated isolation wards.

Infection-control measures in the non-SARS area were upgraded stepwise in response to possible healthcare-associated transmission and the increasing possibility of community spread of SARS. After the outbreak in hospital A, healthcare workers wore N95 respirators for all patient care in the ER. In the ER, SARS areas were cleaned at least three times; non-SARS areas were cleaned once a day.

Identifying Cases and Sources of Exposure

We obtained source and contact information for all patients identified at our hospital as having suspected or probable SARS. After the outbreak in hospital A, health insurance records were used to trace prior visits to other hospitals. In addition, we obtained information about social, hospital, and occupational contacts and other members of the household who had been exposed to suspected SARS patients within 10 days before the onset of their symptoms. All close contacts exposed to SARS patients during the period from 2 days before the onset of fever to the time of isolation were traced to identify the need for quarantine. Inpatients who had close contact with SARS patients were quarantined in private rooms, and contact and droplet precautions were implemented. On April 30, because of the occurrence of two closely spaced cases of SARS in the ER observation unit (a non-SARS area), we immediately identified a potential outbreak. Accordingly, we screened all inpatients that had been admitted through the ER and telephoned all the patients who stayed in the observation unit from April 23 through April 29.

Soon after the cluster of SARS was identified among healthcare workers on May 12, we devised a questionnaire to identify the source and the factors contributing to infection. Data about daily exposures were collected from April 30 through May 12, 2003. These data included contact with SARS patients; work areas; day, time, and characteristics of duty; exposure to high-risk aerosol-generating procedures; use of personal protective equipment; hand hygiene practices; and contact with other healthcare workers who did not use N95 respirators.

Environmental Survey

On May 15, surfaces of environment and equipment were sampled with moistened sterile cotton swabs. The swabs were spread immediately onto 3 mL of viral transport medium. Samples were collected from various objects in different areas of ER.

Air samples were taken with both high-volume and low-volume samplers in 10 locations in the ER. We used a high-volume air sampler (XMX Virtual Impactor, Dycor Technologies Ltd., Alberta, Canada) to draw air at calibrated sampling rates of 400 L per min for 5 min into a collector with 5 mL of phosphate-buffered saline (PBS). We also used a low-volume pump (Sidekick, SKC Inc., Eighty Four, PA) to draw air at a calibrated sampling rate of 2.0 L per min for 10 h onto a 37-mm diameter, 0.3-μm pore size polytetrafluoroethylene membrane filters. The collected samples were then frozen at –70°C before RNA extraction. Once environmental contamination was identified, cleaning was performed. Follow-up surveillance for contaminated objects was conducted on May 25.

Viral Molecular Testing

Swab samples were suspended in 5 mL of PBS or 3 mL of viral transport medium. Total RNA from 140 μL of the sample was extracted by using a QIAamp Virus RNA Mini Kit (Qiagen, Hilden, Germany) and eluted in 60 μL of buffer. A volume of 5 μL of RNA solution was analyzed. RealArt HPA-Coronavirus LC RT-PCR Reagents (Roche, Penzberg, Germany) were used for one-step real-time reverse transcription–polymerase chain reaction (RT-PCR) in the Roche LightCycler Instrument (Roche, Mannheim,

Germany). This ready-to-use system is designed for specific amplification of the 80-bp region of the SARS-CoV genome and for directly detecting the specific amplicon in fluorimeter channel F1 of the LightCycler Instrument. In addition, these reagents contain a second heterologous amplification system to identify possible PCR inhibition. Internal controls in each run of the experiment included two negative controls (one for RNA extraction and one for RT-PCR) and four quantification standards (1 x 10^1 copies/μL, 1 x 10^2 copies/μL, 1 x 10^3 copies/μL, and 1 x 10^4 copies/μL). We used the following formula to convert the values determined by using the standard curve into copies per milliliter of sample material: results (copies/mL) = result (copies/μL) x elution volume (μL)/sample volume (mL). Data were presented as number of copies per sample.

Results

Identification of Outbreaks in the ER

From March 15 through April 22, a median of 6 patients per day (range 0–29) were screened at the ER for febrile illnesses (Figure 1). After the outbreak in hospital A, a median of 36 patients per day (range 21–67) were screened. Thus, the ER was used to screen a large portion of persons during this rapidly progressing epidemic. Of 754 patients screened at the ER from April 23 through May 12, a total of 63 patients were identified as SARS cases and were admitted to National Taiwan University Hospital, 68 SARS patients were transferred to another hospital, and 155 received care in a temporarily designated ER area because of shortages of isolation rooms and staff. On May 7, up to 18 SARS patients stayed in the ER overnight. Of 232 SARS patients admitted to the National Taiwan University Hospital from March 14 through June 19, 31 (13.4%) did not have a history of travel, exposure to SARS patients, or a hospital visit within 10 days before illness, and the only contact history was a stay at the National Taiwan University Hospital ER.

Source and Contact Tracing

We identified three distinct clusters by plotting the dates of onset of fever for each case (Figure 2) and allocation of bed numbers in the observation unit of patients involved (Figure 3). The first cluster of five patients had disease onset from April 29 through May 1; the second cluster of 14 cases began on May 4, and the third cluster of 12 cases began on May 11, 2003. In the third cluster, all the cases were in healthcare workers. The first cluster affected patients located in three neighboring beds in the observation unit of the ER (Figure 3). The second cluster affected patients located in four nearby beds and a fifth bed that was >3 m away. The distance between beds was approximately 1 m. None of the cases occurred in beds 9–18, which are separated by a half wall.

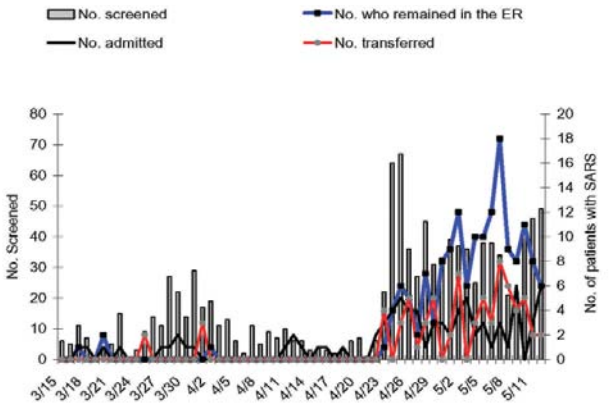

Figure 1. Time course during which patients with febrile illnesses were screened for severe acute respiratory syndrome (SARS) (vertical bars) and patients with SARS were detected at the emergency room of National Taiwan University Hospital, March 15–May 12, 2003. The numbers of patients with SARS who were admitted to this hospital is shown in black lines. The number of patients who temporarily stayed in the emergency room or were transferred to other hospitals is shown in red and blue lines, respectively.

The index patient in the first cluster was an afebrile 73-year-old man who was admitted to the ER because of severe dyspnea (Figure 4, 1P). He was kept in the observation unit from April 23 through April 25, 2003. He was thought to have congestive heart failure and chronic obstructive lung disease and treated with aerosolized medication. He was admitted to the cardiology ward on April 25. A temperature of >38°C developed on April 27, and a chest radiograph taken on the same day indicated a new infiltrate. He was immediately transferred to a negative-pressure isolation room. He had not given this history, but after checking his health insurance card, we learned that he had visited hospital A on April 14 and April 15. He died on April 30. Sputum samples were positive for SARS-CoV

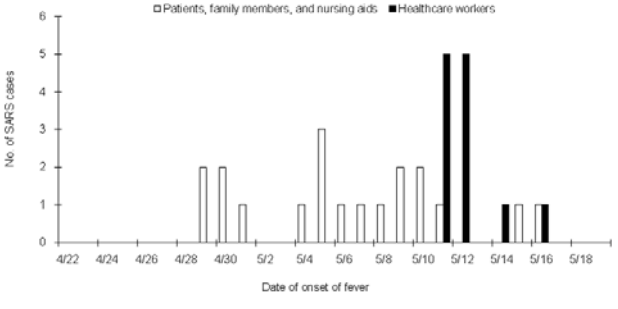

Figure 2. Epidemic curves showing three clusters of cases of severe acute respiratory syndrome (SARS) during the outbreak at the emergency room of the National Taiwan University Hospital. The first two clusters (open bars) consisted of patients, family members, and nursing aids. The third cluster (solid bars) consisted entirely of healthcare workers.

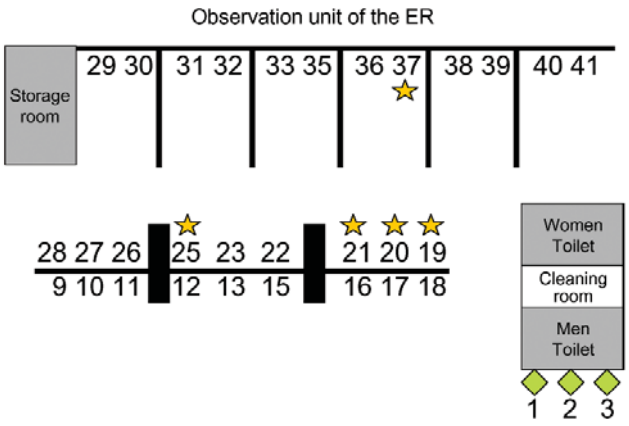

Figure 3. Allocation of bed numbers in the observation unit of patients involved in the first cluster (squares) and the second cluster (stars) of severe acute respiratory syndrome at the emergency room (ER) of National Taiwan University Hospital.

RNA. On autopsy, he was found to have had an acute myocardial infarction. A small ground-glass density in the lung was compatible with viral pneumonitis.

The second patient was another 73-year-old man who had cough and fever for 2 weeks. He had been treated in the ER observation unit from April 24 through April 29. He returned to the ER on April 30 and was diagnosed with probable SARS. Because of the occurrence of two closely spaced cases of SARS in the ER observation unit (a non-SARS area), we immediately identified a potential outbreak. Contact tracing identified a cluster of cases in three patients and two nursing aids (Table 1, patient 2–6; Figure 4). All five cases were diagnosed as probable SARS, and results of three tests were positive for SARS-CoV RNA. The contacts were quarantined, and no tertiary cases emerged.

The second cluster began on May 8. A 46-year-old, otherwise-healthy woman (patient 8) was admitted with probable SARS. A week earlier she had taken care of her mother (patient 9) in the ER observation unit. She indicated that she did not have contact with other SARS patients, including those identified in the first cluster. Accordingly, we screened all patients who stayed in the observation unit from April 30 through May 8. This cluster affected six patients, three family members, and five nursing aids (Figures 2 and 4; Table 1, patients 7–20). Patient 17 was the only tertiary case.

The third cluster was noted on May 12, when the infection control team was informed that fever developed in three healthcare workers who had been isolated. The exact contact source could not be identified. Thus, we quarantined all the ER healthcare workers and suspended ER operations for 2 weeks. SARS related to the ER developed in 12 healthcare workers from May 11 through May 16 (Figure 2, solid lines). Six of the healthcare workers who became ill had close contact with SARS patients. However, patient contact and time of exposure were different. The healthcare workers were one desk clerk, two physicians, one radiology technician, and two nurses. All had followed infection-control precautions. Six other healthcare workers who became ill indicated that they did not have close contact with SARS patients. These workers were four nurses and two cleaners. These 12 healthcare workers differed from each other according to duty pattern, service time, work areas, and time of exposure to the unit (data not shown). Source and contact tracing failed to identify a common source. We therefore postulated that they might have acquired SARS through indirect contact.

Environmental Survey

On May 15, we collected 119 environment samples, including 100 surface samples and 19 air samples (Table 2). Nine samples were positive for SARS-CoV RNA. These included the buttons of the drinking water fountains in the triage and the observation unit; a bedside chair in the observation unit; the outlet of the central air supply, a table top, bedding and bed edge in a SARS area; and a bookshelf and bedding in the clean area. None of 19 air samples tested positive for viral RNA. The highest viral load was obtained from a bedside chair in the observation unit (2,570 to 25,700 copies per sample).

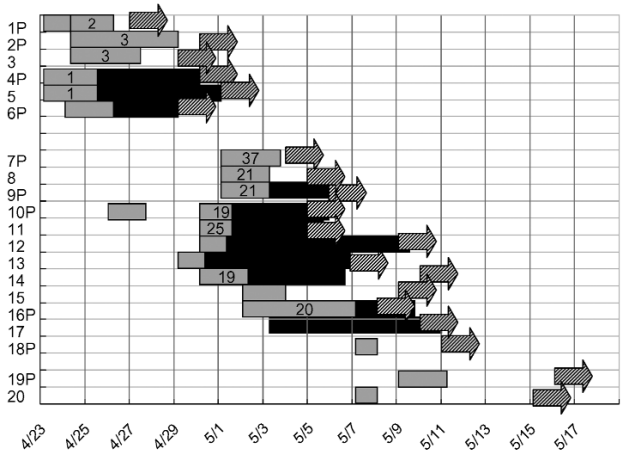

Figure 4. Contact history and temporal relationships among contacts according to the date of fever for 19 cases of severe acute respiratory syndrome (SARS) during the first two clusters of SARS at the emergency room of National Taiwan University Hospital. On April 27, fever and pneumonia developed in the index patient (patient 1) of the first cluster. The second cluster from an unknown source was identified on May 8. P, patients. Unlabeled numbers indicate family members or nursing aids. Location in the emergency room is shown in gray bars and in general wards by black bars. Numbers within the gray bar represent bed numbers in the observation unit of the ER. The dates of onset of fever are shown in arrows.

Table 1. Medical histories and conditions of the index patient and 19 patients affected in the clusters of severe acute respiratory syndrome related to the emergency room (ER) of National Taiwan University Hospital

Patient no.	Age/sex	Characteristics
Index patient		
1	73/M	Coronary artery disease, recent percutaneous occlusive balloon angioplasty and stenting, congestive heart failure, chronic obstructive lung disease, diabetes mellitus, chronic renal insufficiency. Had dyspnea without fever.
First cluster		
2	73/M	Infarction, hypertension, diabetes mellitus, old cerebral vascular accident, parkinsonism, hyperthyroidism. Cough for 2 weeks and fever for 1 week and was treated for aspiration pneumonia.
3	24/F	Nursing aide of patient 2.
4	62/F	Hepatitis C virus–related cirrhosis of liver, hypertension, diabetes mellitus. Had upper gastrointestinal bleeding.
5	64/F	Nursing aide of patient 4. Bronchial asthma.
6	65/F	Common bile duct stone and obstructive jaundice status post endoscopic retrograde cholangiopancreatography and endoscopic nasobiliary drainage, gallbladder stone status post cholecystectomy. Had fever and abdominal pain.
Second cluster		
7	88/M	Chronic obstructive pulmonary disease, hypertension, coronary artery disease status post percutaneous transluminal coronary angiography. Had lower intestinal bleeding secondary to ischemic colitis.
8	46/F	Family member of patient 9.
9	71/F	Acute pancreatitis, diarrhea.
10	65/F	Coronary artery disease status post percutaneous transluminal coronary angioplasty, major depression, diabetes mellitus, hypertension, end-stage renal disease under regular hemodialysis at a regional hospital. Persistent fever, diarrhea, leukocytosis, and normal chest radiograph result.
11	63/F	Took care of her son with acute pancreatitis in the ER.
12	38/M	Took care of his mother with end-stage renal disease undergoing hemodialysis. Stayed in the ER for 2 hours on April 29. Fever developed on May 7. Chest radograph findings were abnormal on May 11.
13	48/F	Nursing aide of a patient with pancreatitis close to patient 10 who had frequent diarrhea. She helped take care of patient 10.
14	43/F	Nursing aide of patient 10.
15	24/F	Nursing aide of a patient who visited the ER on May 2.
16	46/F	Pancreatic cancer with liver metastasis, perforated gastric ulcer status post primary closure and duodenostomy, gastrostomy and jejunostomy, poor control of diabetes mellitus and hypertension, glaucoma. Abdominal pain and watery diarrhea and was diagnosed as adhesion ileus and subcutaneous abscess caused by *Klebsiella pneumoniae*.
17	43/F	Nursing aide. Contact of patient 9. Colon tubular adenoma status post polypectomy, chronic paranasal sinusitis status post functional endoscopic sinus surgery.
18	28/M	Tinea pedis, cellulitis.
19	69/M	Coronary artery disease, status post percutaneous transluminal coronary angioplasty. Abdominal discomfort and loss of appetite for several weeks. Cholangiocarcinoma, obstructive jaundice, biliary tract infection, and upper gastrointestinal bleeding were diagnosed.
20	28/F	Nursing aide.

Control Measures and Follow-up

Targeted cleaning of the ER environment was performed. Follow-up surveillance was conducted on May 25. Nine samples were collected from previously contaminated surfaces, 21 samples from other areas in the ER, and 15 samples from SARS wards. All 45 samples were negative for SARS-CoV RNA. All personnel who had contact with SARS patients or their environments were reeducated on infection-control measures. Particular attention was paid to hand hygiene and routine environmental cleaning. The workload for healthcare workers was reduced. All patient beds were placed at least 2 m apart. No further cases of SARS related to the ER occurred after May 17, 2003.

Discussion

This report describes three clusters of SARS cases related to exposure to the ER at National Taiwan University Hospital during the epidemic in Taiwan. The index patient had been exposed to SARS at a nearby hospital. The patients symptoms were atypical for SARS, and he initially indicated that he had not been to hospital A. He had chronic cardiac and pulmonary disease and was afebrile. To date, healthcare-associated acquisition of SARS has been reported from eight hospitals in Taiwan (including National Taiwan University Hospital). All have been linked to the initial outbreak at hospital A (9). Unrecognized cases of SARS are probably the most important factor that led to intrahospital spread and cases among healthcare workers (11,12).

Most patients appear to have acquired their infections by close patient contact, presumably by droplet transmission. Six of the cases among the healthcare workers had no direct SARS patient contact. They may have acquired their infection from commonly used, contaminated objects. Finding SARS-CoV RNA in nine commonly used inanimate objects supports this notion. Although the signal only

Table 2. Results of environmental surveillance for severe acute respiratory syndrome (SARS) coronavirus RNA determined by real-time reverse transcriptase–polymerase chain reaction

Source of samples	No. of samples collected	No. (%) of positive samples	Source of positive result (copies of viral RNA per sample)
Surface of environment			
Triage	11	1 (9.1)	Button of drinking water fountains (257–2,570)
Examination area	10	0	
Observation unit	42	2 (4.8)	Button of drinking water fountains (257–2,570) Bedside chair (2,570–25,700)
Critical care area	3	0	
SARS area	10	4 (40.0)	Outlet of central air supply (257–2,570) Table top (257–2,570) Bedding (257–2,570) Bed edge (257–2,570)
Clean area	14	2 (14.3)	Book shelf (257–2,570) Bedding (257–2,570)
High-efficiency particulate air filter	10	0	
Air			
High-volume sampler	9	0	
Low-volume sampler	10	0	
Total	119	9 (7.6)	

demonstrated SARS-CoV RNA and not viable virus, this finding may indicate that the virus can persist in the environment.

Environmental contamination was first demonstrated during a community outbreak in Hong Kong (13). The SARS virus may be stable in the environment at room temperature for 1 to 2 days (8). It can survive on plastic surfaces, stainless steel, glass slides, and paper files. The virus can survive even longer (up to 4 days) in stool from patients with diarrhea (8). In some series, diarrhea is a common complaint of SARS patients (14). One patient (patient 2) in the first cluster had intestinal bleeding, and 4 of 14 patients in the second cluster had diarrhea.

Overcrowding in the ER during an epidemic creates more opportunities for cross transmission and environmental contamination. In addition, overworked medical staff may not follow preventive procedures and take inadequate precautions (15,16). After the outbreak in hospital A, healthcare workers in the ER wore N–95 respirators for all patient care. Using protective equipment may account for the absence of cases among healthcare workers during the first and the second clusters of SARS in the ER. However, the third cluster included six healthcare workers who were not exposed to patients with SARS. Thus, masks do not prevent acquisition from environmental sources. Furthermore, the spread of SARS was most likely facilitated by lack of proper handwashing than by direct contact with patients or environments contaminated with viral nucleic acids. Therefore, intensive environmental cleaning should be instituted as soon as a case is identified, particularly for those with diarrhea. In addition, the importance of handwashing cannot be overemphasized.

This study has several limitations. Comprehensive serologic surveys were not conducted among all of the healthcare workers and patients during the outbreak. We may have missed persons with subclinical or mild infections who might have transmitted SARS by person-to-person contact. Viral cultures were not performed on samples taken from inanimate objects. SARS virus detected by RT-PCR may not have been viable.

Epidemiologic data suggest that transmission of SARS is mainly through close contact with droplets or secretions. But increasingly epidemiologic evidence, including this report, shows that the disease may also be transmitted indirectly through contact with hands or objects contaminated with secretions or excreta from patients with diarrhea. Clarifying the route of transmission will help prevent nosocomial transmission and allay fears that protection is inadequate.

Acknowledgments

We are grateful to Daniel B. Jernigan, S.Y. Chen, L.H. Wang, and S.H. Wang for their important contributions to this investigation, H.M. Tai for technical help, and Calvin Kunin for critical review of the manuscript.

This study was supported by a grant from the National Science Council, R.O.C. (NSC 92-3112-B-002-043).

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Genetic Variation of SARS Coronavirus in Beijing Hospital

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To characterize genetic variation of severe acute respiratory syndrome–associated coronavirus (SARS-CoV) transmitted in the Beijing area during the epidemic outbreak of 2003, we sequenced 29 full-length S genes of SARS-CoV from 20 hospitalized SARS patients on our unit, the Beijing 302 Hospital. Viral RNA templates for the S-gene amplification were directly extracted from raw clinical samples, including plasma, throat swab, sputum, and stool, during the course of the epidemic in the Beijing area. We used a TA-cloning assay with direct analysis of nested reverse transcription–polymerase chain reaction products in sequence. One hundred thirteen sequence variations with nine recurrent variant sites were identified in analyzed S-gene sequences compared with the BJ01 strain of SARS-CoV. Among them, eight variant sites were, we think, the first documented. Our findings demonstrate the coexistence of S-gene sequences with and without substitutions (compared to BJ01) in samples analyzed from some patients.

A novel severe acute respiratory syndrome–associated coronavirus (SARS-CoV) has been implicated as the causative agent of a worldwide outbreak of SARS during the first 6 months of 2003 (1–3). From March 4 to June 18, Beijing had 2,521 cases and 192 deaths from SARS (4). Because of the poor fidelity of RNA-dependent RNA polymerase, genetic variation typically forms a heterogeneous virus pool in RNA virus populations, including coronaviruses such as mouse hepatitis virus (MHV) (5,6). This feature makes viruses highly adaptable and contributes to difficulties in preventing and controlling viral disease. SARS-CoV, a single-stranded RNA virus, has been reported with relatively less variability in analyses of a limited number of viral isolate collections (7–10). Furthermore, no SARS-CoV quasispecies have been documented, as they have been in many other RNA viruses, including hepatitis C virus (HCV) (11), HIV (12), and MHV (6).

During the SARS outbreak in Beijing, 132 SARS patients were hospitalized and treated on our unit at Beijing Hospital, including the first cluster of case-patients in the area (13). To characterize genetic variation among SARS-CoV transmitted in the Beijing area, we sequenced 29 full-length S genes of SARS-CoV from 20 hospitalized SARS patients, since S glycoprotein plays a key role in virus-host interaction and is predicted to be the main target of immune response (14). Samples that were analyzed represented the timespan of the epidemic. To exclude culture-derived artifacts and estimate mutational heterogeneity, viral RNA was directly extracted from raw clinical samples, and a TA-cloning assay was used with direct analysis of reverse transcriptase–polymerase chain reaction (RT-PCR) products. We compared these sequences with all previously documented S-gene sequences of SARS-CoV.

Materials and Methods

Patients and Samples

All patients in the study were hospitalized on our unit with a confirmed diagnosis of SARS. Samples from patients included plasma, throat swab, sputum, and stool; these were stored at –70°C for extraction of viral RNA. A total of 64 RNA samples from 28 SARS-CoV–positive patients (detected by using BNI primers recommended by the World Health Organization [15]) were initially used in S-gene amplification, but only those that generated all six overlapping fragments covering the full-length S-gene sequence (see Nested RT-PCR below and Figure 1) were included in the sequence analysis. As a result, 29 RNA samples from 20 patients were included in the study (Table 1). All patients had received ribavirin and steroid combination therapy.

RNA Extraction

RNA extraction was performed in a biosafety level 3 (P3) laboratory. RNA was extracted directly from plasma samples. Sputum samples were shaken for 30 min with an equal volume of 1.0% acetylcysteine and 0.9% sodium chloride, followed by isolating supernatant by centrifuging (10,000 *g* x 3 min). Throat swab and stool samples were

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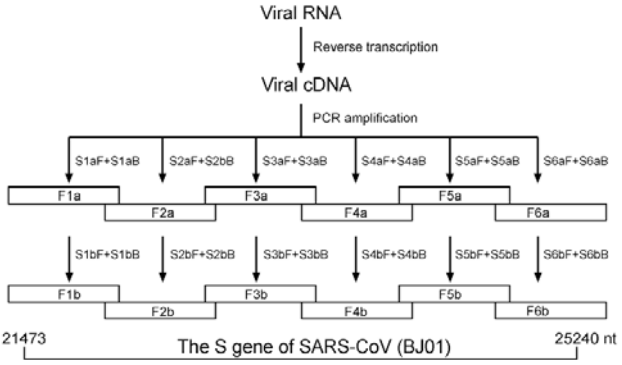

Figure. 1. Diagram showing amplification of six overlapping fragments covering full-length spike gene sequence of severe acute respiratory syndrome–associated coronavirus by nested reverse transcriptase–polymerase chain reaction.

suspended with phosphate-buffered saline (PBS) containing 10 U/mL RNasin (Promega, Madison, WI) and shaken for 10 min, followed by isolating supernatant by centrifuging as mentioned above. RNA was extracted according to the manufacturer's instructions by using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany).

Nested RT-PCR

Screening RNA for SARS-CoV was based on the method by Drosten et al. (1). For the S-gene amplification, 18 pairs of primers were designed by using MacVactor computer software (Accelrys Inc, San Diego, CA) based on the BJ01 strain of SARS-CoV (GenBank accession no. AY278488) (16). Among them, six pairs (sense/antisense: S1aF/S1aB, S2aF/S2aB, S3aF/S3aB, S4aF/S4aB, S5aF/S5aB, S6aF/S6aB) were used as outer primers, six pairs (sense/antisense: S1bF/S1bB, S2bF/S2bB, S3bF/S3bB, S4bF/S4bB, S5bF/S5bB, S6bF/S6bB) were used as inner primers, and six pairs (sense/antisense: S1cF/S1cB, S2cF/S2cB, S3cF/S3cB, S4cF/S4cB, S5cF/S5cB, S6cF/S6cB) were designed for direct RT-PCR product sequencing. The sequences covering the full-length S gene were amplified separately as six overlapping fragments (F1b, F2b, F3b, F4b, F5b, and F6b) (Figure 1). The one-step RT-PCR Kit (Qiagen) was used for reverse transcription and the first round of PCR amplification with outer primers. Thermal cycling consisted of 50°C for 30 min; 95°C for 15 min; 10 cycles of 95°C for 30 s, 57.5°C for 30 s (decreasing by 1.5°C every other cycle), 72°C for 1 min; 40 cycles of 95°C for 30 s, 54°C for 30 s, 72°C for 1 min. Afterwards, 2 μL of the product was used as a template for the second round of PCR amplification in 100-μL

Table 1. Clinical backgrounds of patients and sample collection

Patient no.	Age (y)	Sex[a]	Onset date	Hospitalized date	Specimen no.[b]	Sampling date
1	53	M	2/28	3/05	SW6	3/06
2	32	M	3/08	3/08	SW17	3/09
3	32	F	3/20	4/04	PL1	4/07
4	20	M	3/21	4/06	PL10	4/07
					PL17	4/22
					SP4	5/03
5	33	M	3/28	4/04	PL9	4/07
					SP1	5/03
6	59	M	3/30	4/06	PL5	4/07
					SP9	5/12
7	52	M	3/30	4/04	PL7	4/07
8	59	M	3/30	4/06	PL8	4/07
9	19	F	4/01	4/12	PL15	4/22
					SP32	4/26
10	73	M	4/02	4/03	PL6	4/07
					SP62	4/18
					SW73	4/21
11	45	F	4/04	4/04	SP67	4/18
12	26	M	4/08	4/18	SW76	4/21
13	31	M	4/08	4/14	ST123	4/26
14	32	M	4/09	4/18	PL57	4/21
					SW77	4/22
15	39	M	4/10	4/10	SP61	4/18
16	31	F	4/10	4/12	PL59	4/30
17	46	F	4/20	4/21	SP28	4/26
18	48	M	4/20	4/22	SP43	4/24
19	38	M	4/22	4/26	SP13	5/03
					ST158	4/30
20	27	M	5/10	5/11	SP8	5/12

[a]M, male; F, female.
[b]First two letters indicate source of sample: SW, throat swab; PL, plasma; SP, sputum; ST, stool.

volume with inner primers with Taq DNA polymerase (MBI Fermentas, Hanover, MD). Thermal cycling consisted of 30 cycles of 95°C for 25 s, 54°C for 25 s, 72°C for 50 s. In some cases, superScript III RNase Reverse Transcriptase (Invitrogen, Carlsbad, CA) was used for reverse transcription, according to the manufacturer's instructions. The next two rounds of PCR amplification were performed by using Platinum Pfx DNA Polymerase with a higher fidelity (Invitrogen). The reaction condition was set as above, with a twofold elongation at 68°C instead of 72°C. All reactions were carefully carried out to avoid contamination.

TA-Cloning

RT-PCR products were purified by QIAquick PCR Purification Kit (Qiagen) or QIAquick Gel Extraction Kit (Qiagen), with a final volume of 30 μL of elution. The ligation and transformation were performed according to the manufacturer's instructions by using pGEM-T Vector System II (Promega). Transformants were selected in LB-agar plate containing 100 μg of ampicillin, 100 μg of 5-bromo-4-chloro-3-indolyl β-L-fucopyranoside (X-gal), and 200 μg of isopropylthiogalactoside. *Escherichia coli* from white clones was added to 5 mL of LB culture for overnight growing at 37°C with vigorous shaking. Plasmid was purified by QIAprep Spin Miniprep Kit (Qiagen). The recombinant plasmids for sampling sequence analysis were screened by electrophoresis in 1% agarose containing 0.5 μg/mL of ethidium bromide.

Sequencing and DNA Analysis

For each S-gene fragment, four to six clones were screened. To verify variations, 5–50 additional clones generated from independently prepared, RNA-derived RT-PCR products were sequenced in two to four independent experiments. The cloned plasmids were prepared from different RT-PCR products and were directly sequenced for confirmation. DNA sequences were obtained with the use of an automated ABI 377 sequencer (Applied Biosystems Inc., Foster City, CA). For cloned plasmids, SP6 and T7 primers were used for two-directional sequencing reactions. For PCR products, specific primers (sense: S1cF–S6cF; antisense: S1cB–S6cB) were used for two-directional sequencing reactions. Analysis and comparison of nucleotide and amino acid sequences were carried out with the DNASTAR computer software (DNASTAR Inc., Madison, WI). The S gene sequence of BJ01 strain was taken as the reference for variation analysis.

Results

With the designed six pairs of primers, all six overlapping S-gene fragments were amplified by nested RT-PCR from 29 RNA samples. However, most RNA samples initially included in the study, though positive for SARS-CoV with BNI primers, failed to simultaneously generate all six overlapping S-gene fragments and were excluded from further sequence analysis. Disintegration of the virus and low viral load in the raw samples likely accounted for these failures.

One hundred and thirteen sequence variations distributed in nine variant sites were identified in analyzed sequences that were compared to the reference BJ01 strain of SARS-CoV. BJ01 is an isolate from a tissue-culture propagated sample (16) and is used as reference strain in other studies (9,10). With the exception of one site (position 21702), other variant sites have not, to our knowledge, been documented in humans. Seven of nine variant sites were nonsynonymous. Figure 2 shows the identified variant sites compared to the reference sequence.

Discussion

We identified novel variant sites and the coexistence of sequences with and without S-gene substitutions in SARS-CoV. Theoretically, a replicating RNA virus expresses a range of genetic and phenotypic variants and has the potential to generate novel virions, which may be selected in response to environmental pressures. RNA viruses generally tolerate high levels of mutagenesis because of their limited genetic complexity (17). Mutations have the potential to be pathogenic (e.g., giving the virus immunity to neutralizing antibodies, cytotoxic T cells, or antiviral drugs [18–20]). The dynamics of error copying and sequence decomposition are time-dependent. In HIV infection, for example, one adaptive substitution in the *env* gene

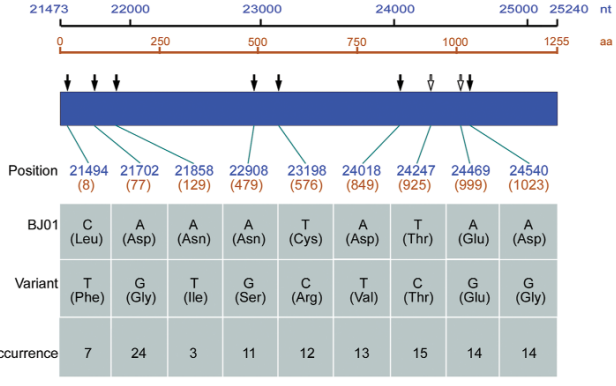

Figure 2. Variants identified from 29 full-length S genes of severe acute respiratory syndrome–associated coronavirus from 20 SARS patients in comparison with BJ01 strain (GenBank accession no. AY278488). The nucleotide positions are numbered according to the sequence of BJ01 strain. Numbers start from the beginning of the genome, but the amino acid numbers start from the S protein. The filled arrows represent nonsynonymous mutations, and the hollow arrows represent synonymous ones. The occurrence indicates the frequency of the variant nucleotide at the given site of the identified 29 entire S genes.

occurred every 3.3 months or 25 viral generations, averaging across patients (21).

In our study, a higher variation frequency in the S gene was identified for SARS-CoV compared to previous reports (7–10). This difference may be due to a broader sample collection covering a longer timespan of infection. In addition, since virus isolates were not passaged in culture, the whole mutant repertoire is more likely to be detected, since no reverse mutation occurs in cell culture. Our observation most likely reflected the real situation in vivo. Variations were unlikely to result from Taq polymerase errors, since we repeated the experiments for all variations from preparing independent RNA and RT-PCR products and used Platinum Pfx DNA polymerase, which has a high fidelity, to confirm the results in some cases. We could not exclude the possibility that some variations were from defective genomes. However, the fact that the variations remained detectable in the sequences from two or three specimens of the same patient, obtained at different times, suggested that these variations might be active and extensible in vivo.

Sequences with and without substitutions (compared to BJ01) were simultaneously detected in the sequences from seven samples, which suggests the existence of SARS-CoV quasispecies. Furthermore, S-gene sequences from different samples collected at different times from the same patient showed similar, but not exactly identical, variation profiles in four participants (patients 4, 5, 6, and 19 in Table 1); this implies that a dynamic mutational process may exist in vivo. Table 2 summarizes the variations occurring in 29 analyzed S-gene sequences from 20 individual SARS patients.

One nonsynonymous change observed at position A1023G is within the heptad repeat (HR) domains, which is thought to be important for virus entry, and previous study on MHV showed that it would have some effect on virus infection (22). At this stage, we cannot rule out the possibility that this change affects the biological outcome

Table 2. Variation in S-gene sequences from 20 individual SARS patients[a,b]

		21494	21702	21858	22908	23198	24018	24247	24469	24540
Pt. no.	Samp. no.	C→T	A→G	A→T	A→G	T→C	A→T	T→C	A→G	A→G
1	SW6	–	–	–	–	–	–	–	–	–
2	SW17	9/2[c]	+	–	–	–	–	+	–	–
3	PL1	8/39	8/43	48/2	+	+	+	+	+	+
4	PL10	14/7	+	–	+	+	2/8	+	+	+
	PL17	+	+	–	–	–	+	+	+	+
	SP4	–	+	–	+	–	+	+	+	+
5	PL9	+	+	–	+	+	+	+	+	+
	SP1	–	+	–	+	+	–	+	+	+
6	PL5	–	+	–	+	+	8/4	+	+	+
	SP9	–	+	–	+	+	+	+	+	+
7	PL7	–	+	–	+	+	4/6	+	+	+
8	PL8	7/28	+	33/2	+	+	+	+	+	+
9	SP15	–	+	–	–	–	–	–	–	–
	SP32	–	+	–	–	–	–	–	–	–
10	SP6	–	–	–	–	–	–	–	–	–
	SP62	–	–	–	–	–	–	–	–	–
	SW73	–	–	–	–	–	–	–	–	–
11	SP67	–	+	–	–	–	–	–	–	–
12	SW76	–	+	–	–	–	–	–	–	–
13	ST123	–	+	–	–	–	–	–	–	–
14	PL57	–	+	–	–	+	+	+	+	+
	SW77	–	+	–	–	+	+	+	+	+
15	SP61	–	+	–	–	–	–	–	–	–
16	PL59	–	–	–	–	–	–	–	–	–
17	SP28	–	+	–	–	–	–	–	–	–
18	SP43	–	+	–	–	–	–	–	–	–
19	ST158	–	+	–	+	+	+	+	+	+
	SP13	19/4	14/10	10/13	+	+	+	6/16	+	14/8
20	SP8	–	+	–	–	–	–	–	–	–

[a]The results were determined by analysis of cloned sequences; + represents that nucleotide substitution at the variant site is detected and – represents that the nucleotide at the site is identical to the one of BJ01 reference sequence in all analyzed sequences.

[b]SARS, severe acute respiratory syndrome; SW, throat swab; PL, plasma; SP, sputum; ST, stool.

[c]The numbers represent the ratio of reference to variant nucleotide detected at the site from the analyzed cloned sequences.

of the virus, but further experiments need to be addressed in the near future.

We observed the coexistence of the S-gene sequences with and without substitutions and time-dependent variation profile in some patients. These observations suggest the possible existence of SARS-CoV quasispecies in an acute infection. In this study, however, the limitation of clinical sample collection and difficulty in directly amplifying full-length S gene from raw clinical samples restricted further extensive study for dynamic mutant distributions of the virus. In addition, the sequencing clone number was conditioned by the scale of the project, and this may have led to some minor variant sequences escaping analysis. Another factor possibly affecting the stability of the viral genome is the administration of the antiviral drug ribavirin. That ribavirin enhances mutagensis of RNA viruses has been addressed (23). Therefore, the artificial effect of ribavirin on the SARS-CoV mutant spectrum remains to be clarified.

The genetic variation of SARS-CoV remains limited in relation to many other RNA viruses such as HIV-1, HCV, and MHV. The probable reason is that SARS-CoV only causes an acute, self-limited infection, which may prevent persistent long-term mutant development in vivo as occurs in chronic RNA viral infections. Notably, some modules in the S protein remain conserved, e.g., the fusion-important HR domains. Although some variations may predict changes of protein functional features, no obvious correlation exists between mutation and clinical disease manifestation from the limited data reported here. Instead, the variation profile was closely correlated with epidemiography; e.g., patients 3–8 were infected in one hospital.

In conclusion, we report here some new variant sites in the S gene of SARS coronavirus and possible existence of SARS-CoV quasispecies in some patients, though in limited numbers. This knowledge furthers our understanding of this emerging virus.

Acknowledgments

We thank K.Y. Yuen for his valuable suggestions for this project; and Panyong Mao and Yuanli Mao for providing some of the samples.

The work was supported by Beijing Natural Science Foundation (Number: 7034051), Emergent Foundation for SARS Treatment and Prevention (Number: 03F017), and in part by Sino-UK Collaboration Foundation for SARS Immunopathogenesis Study (Number: H030230100130).

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Multidrug-resistant Strains of *Salmonella enterica* Typhimurium, United States, 1997–1998[1]

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To evaluate multidrug-resistant strains of *Salmonella enterica* serotype Typhimurium, including definitive type 104 (DT104) in the United States, we reviewed data from the National Antimicrobial Resistance Monitoring System (NARMS). In 1997 to 1998, 703 (25%) of 2,767 serotyped *Salmonella* isolates received at NARMS were *S.* Typhimurium; antimicrobial susceptibility testing and phage typing were completed for 697. Fifty-eight percent (402) were resistant to ≥1 antimicrobial agent. Three multidrug-resistant (≥5 drugs) strains accounted for (74%) 296 of all resistant isolates. Ceftriaxone resistance was present in 8 (3%), and nalidixic acid resistance in 4 (1%), of these multidrug-resistant strains. By phage typing, 259 (37%) of *S.* Typhimurium isolates were DT104, 209 (30%) were of undefined type and 103 (15%) were untypable. Fifty percent (202) of resistant (≥1 drug) isolates were DT104. Multidrug-resistant *S.* Typhimurium isolates, particularly DT104, account for a substantial proportion of *S.* Typhimurium isolates; ceftriaxone resistance is exhibited by some of these strains.

Salmonella enterica serotype Typhimurium is the most common *Salmonella* serotype in the United States, accounting for 29% of the approximately 30,000 laboratory-confirmed *Salmonella* infections reported annually to the Centers for Disease Control and Prevention (CDC) from 1968 to 1998 (1). Among *Salmonella* serotypes, Typhimurium exhibits one of the highest prevalences of antimicrobial resistance (2,3). Of particular concern is a multidrug-resistant strain of *S.* Typhimurium defined by phage typing as definitive type 104 (DT104). Multidrug-resistant DT104 was first detected in the United Kingdom in 1984 and was first isolated in the United States in 1985 (4,5). In addition to the phage reactions, this strain is characterized by its multiple antimicrobial-resistance pattern to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (R-type ACSSuT). The number of reported human isolates of DT104 R-type ACSSuT in the United Kingdom increased from 259 isolates in 1990 to 4,006 isolates in 1996 (6).

In the United States, antimicrobial susceptibility testing determined that the proportion of *S.* Typhimurium isolates that were R-type ACSSuT increased from <1% in 1980 to 34% in 1996 (7). Although phage typing of *S.* Typhimurium isolates is not routinely done in the United States, 93% of the R-type ACSSuT isolates tested from a national sample of isolates from all state and public health laboratories conducted in 1995 were DT104, which suggests that 9% of all human *Salmonella* infections in this country in 1995 were caused by *S.* Typhimurium DT104 R-type ACSSuT (5,7).

The objectives of this analysis were to determine the antimicrobial-resistance patterns seen among *S.* Typhimurium isolates received at CDC through the National Antimicrobial Resistance Monitoring System (NARMS) from 1997 through 1998 and describe the distribution of phage types, including DT104, among *S.* Typhimurium isolates with the most common resistance patterns.

Methods

In 1996, NARMS was established to prospectively monitor the patterns of antimicrobial-drug resistance among human enteric pathogens, including nontyphoidal *Salmonella* isolates received at select public health laboratories in the United States (8). NARMS began as collaboration between CDC, the U.S. Food and Drug Administration Center for Veterinary Medicine, and 12 state health departments (California, Colorado, Connecticut, Florida, Georgia, Kansas, Massachusetts,

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[1]Presented in part at the International Conference on Emerging Infectious Diseases, July 2000, Atlanta, Georgia, USA.

Minnesota, New Jersey, Oregon, Washington, and West Virginia) and two local health departments (Los Angeles County and New York City). Two additional state health departments (Maryland and New York) joined NARMS in 1997. According to 1998 U.S. postcensus estimates (available from http://www.census.gov/population/estimates/state), the population served by these 16 state and local health departments was approximately 97 million persons, 37% of the U.S. population.

From 1997 through 1998, NARMS-participating public health laboratories forwarded every 10th nontyphoidal *Salmonella* isolate serotyped at their laboratory to CDC for susceptibility testing. At CDC, partial range MICs were determined by using broth microdilution (Sensititre, Trek Diagnostics, Westlake, OH) for 16 antimicrobial agents: amikacin, amoxicillin-clavulanic acid (Cl), ampicillin (A), apramycin (Ap), ceftiofur (a third-generation cephalosporin used in veterinary medicine) (Cef), ceftriaxone (Cx), cephalothin (Cep), chloramphenicol (C), ciprofloxacin, gentamicin (G), kanamycin (K), nalidixic acid (N), streptomycin (S), sulfamethoxazole (Su), tetracycline (T), and trimethoprim-sulfamethoxazole (Tm) (NARMS 1997, 1998 Annual Reports; available from http://www.cdc.gov/narms). National Committee for Clinical Laboratory Standards (NCCLS) interpretive criteria were used when available (9); resistance to ceftiofur, apramycin, and streptomycin was defined as an MIC ≥8 μg/mL, ≥32 μg/mL, and ≥64 μg/mL, respectively. Isolates that exhibited decreased susceptibility to third-generation cephalosporins (ceftiofur or ceftriaxone) were confirmed as *Salmonella* and tested for the full range of MICs for ceftriaxone by broth microdilution using NCCLS standards and by further molecular characterization (10,11). All isolates with intermediate susceptibilities were categorized as susceptible for this analysis with the understanding that an intermediate susceptibility to some drugs, in particular, ceftriaxone, would remove this drug as a clinical option.

Phage types were determined by using a scheme of 31 *S.* Typhimurium typing phages based on the method of Anderson et al. (12) and the interpretive guide supplied by the Public Health Laboratory Service (PHLS) in Colindale, United Kingdom. At the time this set of *S.* Typhimurium isolates was tested, additional *S.* Typhimurium typing phages 1, 2, 3, and 18 (which would have enabled designation of definitive types 193, 194, 195, and 208) were not used. *S.* Typhimurium isolates phage typed as definitive type 104, 104a, 104b, 104c, or U302 (closely related definitive types) were classified together as DT104 complex (hereafter referred to as DT104). Those *S.* Typhimurium isolates that reacted to phages but did not conform to any defined pattern were classified as RDNC (reacts but does not conform), and those that did not react with any of the typing phages used at the time were classified as untypable. Isolates that did not have antimicrobial susceptibility test results or phage type results were excluded from analysis. Invasive isolates were classified as those isolated from specimens collected from normally sterile sites, such as blood or cerebral spinal fluid; enteric isolates were those isolated from stool specimens or rectal swabs. Isolates from specimens collected from other (e.g., urine) or unknown sources were excluded from analysis by specimen source. Statistical analysis was performed with Epi Info 6.04 (CDC, Atlanta, GA) and SAS 6.12 software (SAS Institute Inc., Cary, NC). Statistical testing of differences in proportions was conducted using the chi-square test; p values <0.05 were considered significant.

Results

Resistance Testing

A total of 2,767 serotyped nontyphoidal *Salmonella* isolates were received at CDC through NARMS from 1997 through 1998; 1,301 in 1997 and 1,466 in 1998. Of these, 703 (25%) were *Salmonella* serotype Typhimurium (including serotype Typhimurium var. Copenhagen); 326 (25%) in 1997 and 377 (26%) in 1998. Antimicrobial susceptibility testing and phage typing was completed on 697 isolates.

The antimicrobial agents to which *S.* Typhimurium isolates demonstrated the highest level of resistance were sulfamethoxazole (53%), streptomycin (51%), tetracycline (50%), ampicillin (48%), chloramphenicol (35%), kanamycin (16%), amoxicillin-clavulanic acid (5%), cephalothin (5%), gentamicin (4%), trimethoprim-sulfamethoxazole (4%), ceftiofur (2%), ceftriaxone (1%), and nalidixic acid (1%). No isolates were resistant to amikacin, apramycin, or ciprofloxacin.

Overall, 402 (58%) *S.* Typhimurium isolates were resistant to ≥1 antimicrobial agent tested, 379 (54%) were resistant to ≥2 antimicrobial agents, and 312 (45%) were resistant to ≥5 antimicrobial agents (Table 1). Three distinct multidrug-resistant patterns were found among the 312 isolates resistant to ≥5 agents: 209 (67%) were resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline (R-type ACSSuT), 26 (8%) were additionally resistant to kanamycin (R-type ACKSSuT) and 61 (20%) were resistant to ampicillin, kanamycin, streptomycin, sulfamethoxazole, and tetracycline (R-type AKSSuT).

The proportion of *S.* Typhimurium isolates that were R-type ACSSuT, R-type ACKSSuT, or R-type AKSSuT varied among the 16 NARMS sites. Among the 14 sites that submitted ≥10 *S.* Typhimurium isolates, New York State had the highest proportion of isolates that were one of these phenotypes (64%), and Minnesota had the lowest (17%) ($p < 0.001$) (Table 2). *S.* Typhimurium R-type ACSSuT was

Table 1. R-type and phage type distribution among *Salmonella* Typhimurium isolates, NARMS 1997–1998

R-type[a]	All isolates, N (%)	DT104, N (%)	RDNC[b], N (%)	Untypeable, N (%)
ACSSuT	187 (27)	160 (62)	6 (3)	6 (6)
ACSSuT+Cl	11 (1)	10 (4)	0	0
ACSSuT+G	2 (<1)	2 (<1)	0	0
ACSSuT+N	4 (<1)	4 (2)	0	0
ACSSuT+Tm	1 (<1)	0	0	0
ACSSuT+Cl+Cep	2 (<1)	1 (<1)	0	0
ACSSuT+Cl+Tm	1 (<1)	1 (<1)	0	0
ACSSuT+Cl+Cef+Cx+Cep	1 (<1)	1 (<1)	0	0
ACSSuT total	209 (30)	179 (69)	6 (3)	6 (6)
ACKSSuT	16 (2)	13 (5)	1 (<1)	2 (2)
ACKSSuT+Cep+Tm	4 (<1)	0	0	4 (4)
ACKSSuT+Cl+Cef+Cx+Cep	3 (<1)	0	0	3 (3)
ACKSSuT+Cl+Cef+Cx+Cep+G	2 (<1)	1 (<1)	0	1 (1)
ACKSSuT+Cl+Cef+Cx+Cep+G+Tm	1 (<1)	0	0	1 (1)
ACKSSuT total	26 (4)	14 (5)	1 (<1)	11 (11)
AKSSuT	51 (7)	5 (2)	8 (4)	34 (33)
AKSSuT+Cep	4 (<1)	1 (<1)	1 (<1)	2 (2)
AKSSuT+G	1 (<1)	0	1 (<1)	0
AKSSuT+Cl+Cep	1 (<1)	0	0	0
AKSSuT+Cep+G	2 (<1)	2 (<1)	0	0
AKSSuT+Cl+Cep+G	1 (<1)	1 (<1)	0	0
AKSSuT+Cef+Cx+Cep+N	1 (<1)	0	0	1 (1)
AKSSuT total	61 (9)	9 (4)	10 (5)	37 (36)
Pansusceptible	295 (42)	33 (13)	144 (69)	26 (25)
Resistant to 1 antimicrobial agent	23 (3)	5 (2)	11 (5)	3 (3)
Resistant to 2 antimicrobial agents	19 (3)	7 (3)	8 (4)	1 (1)
Resistant to 3 antimicrobial agents	21 (3)	4 (2)	10 (5)	4 (4)
Resistant to 4 antimicrobial agents	27 (4)	5 (2)	11 (5)	10 (10)
Resistant to 5 antimicrobial agents	6 (<1)	3 (1)	2 (1)	1 (1)
Resistant to 6 antimicrobial agents	4 (<1)	0	1 (<1)	0
Resistant to 7 antimicrobial agents	5 (<1)	0	2 (1)	1 (1)
Resistant to 9 antimicrobial agents	1 (<1)	0	0	1 (1)
Total	697 (100)	259 (100)	209 (100)	104 (100)

[a]NARMS, National Antimicrobial Resistance Monitoring System; Cl, amoxicillin-clavulanic acid; A, ampicillin; Ceft, ceftiofur; Cx, ceftriaxone; Cep, cephalothin; C, chloramphenicol; G, .gentamicin; K, kanamycin; N, nalidixic acid; S, streptomycin; Su, sulfamethoxazole; T, tetracycline; Tm, trimethoprim-sulfamethoxazole.
[b]Reacts but does not conform.

found in all states, and the proportion of *S.* Typhimurium isolates that were R-type ACSSuT ranged from 4% in Minnesota to 64% in New York State (Table 2). *S.* Typhimurium R-type ACKSSuT isolates were not found in California, New York State, Oregon, or West Virginia. *Salmonella* Typhimurium R-type AKSSuT isolates were not found in New York State, Washington, or West Virginia, and among other sites ranged from 4% in Maryland and Colorado to 20% in Massachusetts (Table 2).

Both R-type ACSSuT and R-type AKSSuT isolates were identified in all months of the 2-year surveillance period. In contrast, R-type ACKSSuT resistance was first noted in isolates collected during June 1997; from this point forward, R-type ACKSSuT isolates were found every month. For each year, the proportion of *S.* Typhimurium isolates that were R-type ACSSuT in the winter (January–March) was 45% (61/135), compared with 20% (41/201) in the summer (July–September) ($p < 0.001$).

The proportion of multidrug-resistant isolates that were R-type ACSSuT varied significantly by age ($p < 0.01$) for the 542 isolates for which patient's age was known. Those from patients 40–49 years of age had the highest proportion of R-type ACSSuT isolates (41%), and those 10–19 years of age had the lowest proportion (19%).

R-type ACSSuT strains were significantly more likely to be isolated from a sterile site ($p < 0.01$) than from stool when compared with other R-types or with pansusceptible *S.* Typhimurium isolates. Nine percent (19/203) of R-type ACSSuT isolates were from blood compared with 3% (9/291) of pansusceptible isolates. This association was not seen with other R-types.

Of the 209 *S.* Typhimurium R-type ACSSuT isolates, 15 (7%) were also resistant to amoxicillin-clavulanic acid, 4 (2%) were resistant to nalidixic acid, 3 (1%) were resistant to cephalothin, 2 (1%) were resistant to trimethoprim, 2 (1%) were resistant to gentamicin, 1 was resistant to ceftiofur, and 1 was resistant to ceftriaxone. Of 26 *S.*

Table 2. *Salmonella* Typhimurium isolates with ACSSuT, ACKSSuT, or AKSSuT resistance patterns by site, NARMS 1997–1998

Site	ACSSuT, N (%)	ACKSSuT, N (%)	AKSSuT, N (%)	Other R-types, N (%)	Pansusceptible, N (%)	Total N
California[b]	8 (32)	0	3 (12)	5 (20)	9 (36)	25
Colorado	16 (36)	2 (4)	2 (4)	8 (18)	17 (38)	45
Connecticut	15 (39)	1 (3)	4 (10)	6 (15)	13 (33)	39
Florida	4 (45)	1 (11)	1 (11)	0	3 (33)	9
Georgia	21 (26)	1 (1)	6 (8)	9 (11)	43 (54)	80
Kansas	2 (11)	2 (11)	1 (5)	5 (26)	9 (47)	19
Los Angeles[c]	15 (25)	3 (5)	7 (12)	18 (31)	16 (27)	59
Maryland	12 (44)	1 (4)	1 (4)	2 (7)	11 (41)	27
Massachusetts	21 (24)	3 (3)	18 (21)	9 (10)	37 (42)	88
Minnesota	2 (4)	2 (4)	5 (9)	12 (23)	32 (60)	53
New Jersey	28 (32)	5 (6)	4 (5)	11 (13)	38 (44)	86
New York City[d]	10 (21)	2 (4)	7 (15)	7 (15)	22 (45)	48
New York State[e]	21 (64)	0	0	2 (6)	10 (30)	33
Oregon	8 (30)	0	2 (7)	2 (7)	15 (56)	27
Washington	25 (48)	3 (6)	0	7 (13)	17 (33)	52
West Virginia	1 (14)	0	0	3 (43)	3 (43)	7
Total	209 (30)	26 (4)	61 (9)	106 (15)	295 (42)	697

[a]NARMS, National Antimicrobial Resistance Monitoring System.
[b]San Francisco and Alameda Counties.
[c]Los Angeles County.
[d]Five counties of New York City (Bronx, Kings, New York, Queens, Richmond).
[e]Excluding New York City.

Typhimurium R-type ACKSSuT isolates, 10 (39%) were resistant to cephalothin, 6 (23%) were resistant to amoxicillin-clavulanic acid, 6 (23%) were resistant to ceftiofur, 6 (23%) were resistant to ceftriaxone, 5 (19%) were resistant to trimethoprim, and 3 (12%) were resistant to gentamicin. Of the 61 *S.* Typhimurium R-type AKSSuT isolates, 9 (15%) were resistant to cephalothin, 4 (7%) were resistant to gentamicin, 2 (3%) were resistant to amoxicillin-clavulanic acid, 1 was resistant to ceftiofur, 1 was resistant to ceftriaxone, and 1 was resistant to nalidixic acid (Table 1).

Although no *S.* Typhimurium isolates were resistant to ciprofloxacin, two isolates had reduced susceptibility to ciprofloxacin (both had MIC = 0.250 μg/mL), and both isolates were R-type ACSSuT. Twelve *S.* Typhimurium isolates had ceftriaxone MICs ≥32 μg/mL; 11 were resistant (MIC ≥64 μg/mL). Nine (82%) of the 11 ceftriaxone resistant isolates were from children ≤18 years of age; 7 were from children ≤6 years of age. As a group, these 11 isolates were among the most highly resistant seen, with 8 isolates (80%) resistant to >9 antimicrobial agents; 6 were R-type ACKSSuT, 1 was R-type ACSSuT, and 1 was R-type AKSSuT.

Phage Testing

Of the 697 *S.* Typhimurium phage typed, 259 (37%) were DT104, 209 (30%) were RDNC, 103 (15%) were untypable; 35 other phage types were identified at low frequency (<3% of total) among the remaining 126 *S.* Typhimurium isolates (Table 3). Among the 295 pansusceptible *S.* Typhimurium isolates, there were 37 different phage types; 144 (49%) were RDNC, 33 (11%) were DT104, 26 (9%) were untypable, 20 (7%) were DT46, 14 (5%) were DT10, and 10 (3%) were DT2 isolates. Among the 296 isolates that were R-type ACSSuT, ACKSSuT, or AKSSuT, seven different phage types were found; 202 (68%) were DT104, 54 (18%) were untypable, 17 (6%) were RDNC, 7 (2%) were DT12/12A, 6 (2%) were DT21, 6 (2%) were DT 110/110B, and 4 (1%) were DT120. All three of these multidrug-resistant *S.* Typhimurium isolates included some DT104 isolates; 179 (86%) of the 209 R-type ACSSuT isolates, 14 (54%) of the 26 R-type ACKSSuT isolates, and 9 (15%) of the 61 R-type AKSSuT isolates were DT104 (Figure). All four of the nalidixic acid resistant R-type ACSSuT isolates were DT104. Two other prevalent phage categories among multidrug-resistant *S.* Typhimurium isolates were RDNC (and did not necessarily exhibit the same lysis pattern) and untypable. Six (3%) of the R-type ACSSuT isolates, one (4%) of the R-type ACKSSuT isolates, and 10 (3%) of the R-type AKSSuT isolates were RDNC. Six (3%) of the R-type ACSSuT isolates, 11 (42%) of the R-type ACKSSuT isolates, and 37 (61%) of the R-type AKSSuT isolates were untypable. Compared with other phage types, DT104 isolates were more likely to be R-type ACSSuT (86% vs. 3%; $p < 0.01$), and untypable isolates were more likely to be R-type AKSSuT (61% vs. 16%; $p < 0.01$).

Discussion

This comprehensive study of phage type and antimicrobial resistance among *S.* Typhimurium isolates in the United States confirms that multidrug resistance is common

Table 3. *Salmonella* Typhimurium isolates by phage type, 1997–1998

Phage type	N (%)
104	168 (24)
104A	24 (3)
104B	27 (4)
104C	4 (1)
U302	36 (5)
DT104	259 (37)
RDNC	209 (30)
Untypeable	103 (15)
1	6 (1)
2	14 (2)
4A	1 (<1)
9	1 (<1)
10	14 (2)
12/12A	10 (1)
21	6 (1)
22	2 (<1)
18	1 (<1)
36	1 (<1)
38	1 (<1)
40/40 VAR	3 (<1)
41/41A	3 (<1)
46	20 (3)
66	2 (<1)
69	2 (<1)
87	1 (<1)
105	1 (<1)
106	3 (<)
107	1 (<1)
110/110B	10 (2)
114	1 (<1)
120	6 (1)
124	2 (<1)
126	5 (<1)
156	3 (<1)
160	2 (<1)
164	1 (<1)
167	1 (<1)
170	1 (<1)
U291	1 (<1)
Total	697 (100)

among *S.* Typhimurium isolates and that DT104 is the dominant phage type. We found that 42% of *S.* Typhimurium collected during 1997–1998 belonged to one of three multidrug-resistant phenotypes: R-type ACSSuT, R-type ACKSSuT, and R-type AKSSuT. Overall, 68% of these isolates belonging to one of the multidrug-resistant phenotypes were DT104, with the greatest proportion of DT104 among the R-type ACSSuT isolates (86%); taken together, 28% of all *S.* Typhimurium isolates were DT104 R-type ACSSuT. Since an estimated 1.4 million persons are infected with *Salmonella* each year in the United States (13), these data suggest that approximately 100,000 persons were infected annually with *S.* Typhimurium DT104 R-type ACSSuT in 1997 and 1998 in this country.

This study also contributes to our understanding of the descriptive epidemiology of *S.* Typhimurium R-type ACSSuT. *S.* Typhimurium R-type ACSSuT isolates were found in all sites with the highest proportion from New York and the lowest from Minnesota. The proportion of *S.* Typhimurium isolates that were R-type ACSSuT increased during the winter and declined during the summer. *S.* Typhimurium R-type ACSSuT isolates were also most likely to be found in persons 40–49 years of age and least likely to be from those 10–19 years of age. *S.* Typhimurium R-type ACSSuT isolates were more likely to be isolated from sterile sites than were other multidrug-resistant or susceptible *S.* Typhimurium isolates. Further studies are needed to determine if *S.* Typhimurium R-type ACSSuT isolates are more invasive than other *S.* Typhimurium isolates.

The other two predominant multidrug-resistant *S.* Typhimurium seen were R-type ACKSSuT and AKSSuT. Those isolates were largely RDNC or untypable by phage typing. At the time phage typing was done, additional typing phages 1, 2, 3 and 18 of the Colindale scheme were not used. These additional typing phages assist in defining what would otherwise be interpreted as untypable. The untypable categories described here could include isolates that would now be designated as DT193, 194, 195, or 208 if these additional typing phages were applied. Multidrug resistance has been described among DT193 and 208 isolates identified in other studies (14,15). DT193 and 208 represented 10.9% of the pentaresistant *S.* Typhimurium from animals submitted to the U. S. National Veterinary Services Laboratory in 1998 (15), and DT193 was also the most prevalent phage type among 155 multidrug-resistant *S.* Typhimurium tested in southern Italy between 1992 and 1997 (16).

The number of phage-untypable isolates within ACKSSuT and AKSSuT resistance patterns raises the

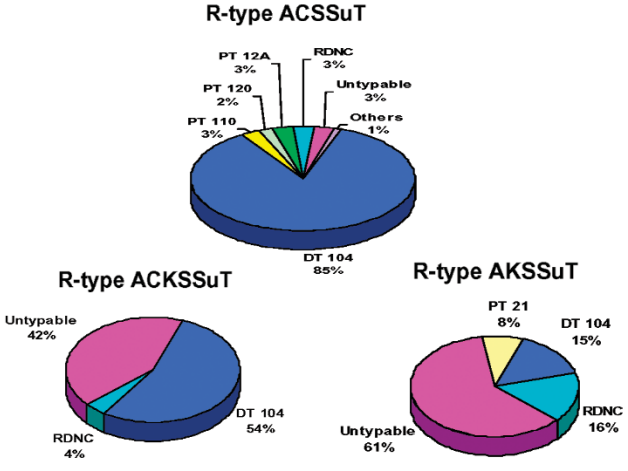

Figure. Distribution of *Salmonella* Typhimurium phage types among resistance patterns.

question of whether multidrug resistance is associated with untypability. Within R-type ACKSSuT, 42% of isolates were untypable and within R-type AKSSuT, 61% were untypable. Transformation of *Salmonella enterica* serotype Enteritidis (*S.* Enteritidis) with drug resistance plasmids has been documented to cause changes in phage type (17,18). Brown et al. (17) showed a shift of PT8 to 13a upon acquisition of IncX plasmid pOG670, which confers resistance to ampicillin and kanamycin. Threlfall et al. (18) reduced susceptibility to phages within the *S.* Enteritidis typing set in several *S.* Enteritidis isolates, and in two cases produced untypable isolates by transforming isolates with an IncN plasmid that contained ampicillin and streptomycin resistance factors. Further work to explore the plasmid and prophage content of isolates from each resistance type, may clarify the importance of these extrachromosomal elements in determinating resistance and phage type.

The emergence of clinically important antimicrobial resistance is cause for concern. Occurrence of third-generation cephalosporin resistance among the multidrug-resistant subpopulation of *S.* Typhimurium isolates is notable. Third-generation cephalosporins (e.g., ceftriaxone) are important in treating invasive *Salmonella* infections, particularly in children (19). In fact, most isolates we describe with ceftriaxone resistance came from children ≤18 years of age. A plasmid-mediated blaCMY-2 mechanism has been described as the source for the expanded-spectrum β-lactam resistance among *Salmonella* that includes ceftriaxone seen in recent years in the United States (10). The prevalence of blaCMY-2 has continued to increase among multidrug-resistant *Salmonella*, especially serotype Newport, in more recent years (20,21). Occurrence of nalidixic acid resistance among multidrug-resistant *S.* Typhimurium R-type ACSSuT DT104 is also notable. Fluoroquinolones are also important in treating invasive *Salmonella* infections, particularly in adults. Although no fluoroquinolone resistant isolates were identified in this study, four R-type ACSSuT isolates were resistant to nalidixic acid; patients with nalidixic acid resistant *Salmonella* DT104 R-type ACSSuT infections have failed treatment with fluoroquinolones (19).

Resistance to multiple antimicrobial agents is common among *S.* Typhimurium. With continued selective pressure that is created by antimicrobial drug use in humans, agriculture, and particularly food animals, we can expect to see a continued high prevalence of multidrug resistance among *S.* Typhimurium. Identification and subtyping of *S.* Typhimurium isolates is essential for understanding and controlling multidrug-resistant *S.* Typhimurium infections. Phage typing provides a useful, although resource-intensive, subtyping tool for this common serotype, although isolates that are RDNC and untypeable need to be categorized into groups that reflect the circulating *S.* Typhimurium strains found in the United States. Continued surveillance of antimicrobial resistance and phage types among *S.* Typhimurium will monitor dissemination of multidrug-resistant strains and strains resistant to clinically important antimicrobial agents, including cephalosporins and fluoroquinolones.

This work was supported in part by the Connecticut Emerging Infections Program and a Cooperative Agreement (U50/CCU111188-07) from the Centers for Disease Control and Prevention.

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Seasonal Forecast of St. Louis Encephalitis Virus Transmission, Florida

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Disease transmission forecasts can help minimize human and domestic animal health risks by indicating where disease control and prevention efforts should be focused. For disease systems in which weather-related variables affect pathogen proliferation, dispersal, or transmission, the potential for disease forecasting exists. We present a seasonal forecast of St. Louis encephalitis virus transmission in Indian River County, Florida. We derive an empiric relationship between modeled land surface wetness and levels of SLEV transmission in humans. We then use these data to forecast SLEV transmission with a seasonal lead. Forecast skill is demonstrated, and a real-time seasonal forecast of epidemic SLEV transmission is presented. This study demonstrates how weather and climate forecast skill-verification analyses may be applied to test the predictability of an empiric disease forecast model.

St. Louis encephalitis virus (SLEV) is a mosquito-borne pathogen that is prevalent throughout much of North America. Florida is subject to periodic outbreaks of SLEV; five epidemics (>20 human clinical cases) have been recorded in south Florida since 1952 (1). The most recent epidemic occurred in 1990 when 226 clinical cases and approximately 30,000 infections were reported throughout south-central Florida. Indian River County was the epicenter of this outbreak (2).

The annual dynamics of SLEV in south Florida can be divided into four phases: January–March maintenance; April–June amplification; July–September early transmission; October–December late transmission (3). The amplification phase involves the epizootic cycling of SLEV between mosquito vectors and avian amplification hosts. Amplification is necessary to achieve mosquito infection rates sufficient to cause human epidemics (4). In Florida, resident juvenile and nestling wild birds are the primary amplification hosts of SLEV (5). Young birds are excellent viral amplification hosts because of their inefficient and poorly developed immune systems, reduced mobility, lack of defense, and their sparse feather coverage, which enables blood-feeding by mosquitoes (5).

Previously, we analyzed historical sentinel chicken seroconversion datasets, i.e., measures SLEV transmission, from 1986 to 1991 in Indian River County (6). Above average seroconversion of sentinel chickens, as measured by serum assay for hemagglutination inhibition (HI) antibodies to SLEV, has been correlated with clinical disease in humans (1). We used a dynamic hydrology model (7) to hindcast mean area water table depth (WTD) in Indian River County for 1986–1991, and compared this model simulation to the sentinel chicken seroconversion data. By using logistic regression, we found the probability of sentinel chicken seroconversion to be strongly associated with low WTD 17 weeks earlier and higher WTD 2 weeks earlier.

A rationale for this empiric relationship was suggested by mosquito collection data, also from Indian River County from 1986 to 1991. *Culex nigripalpus* Theobald is the demonstrated enzootic and epidemic vector of SLEV in south Florida (8–10). Collections of *Cx. nigripalpus* were made in the densely vegetated "hammock" habitats used by this species for daytime resting. During the driest conditions (i.e., modeled WTDs <–1.45 m) preceding heavy SLEV transmission, the numbers of *Cx. nigripalpus* dramatically increased (6) (Figure 1). Rather than indicating an increase in mosquito abundance, these data suggest that drought restricts *Cx. nigripalpus* flight activity to woodland habitats. Extreme droughts in south Florida tend to occur during the spring when nesting wild birds also make use of the hammocks. Thus, drought drives the mosquitoes and birds into contact with one another. This forced interaction of vector mosquitoes and susceptible avian hosts provides an ideal environment for the rapid epizootic amplification of SLEV. Subsequently, when the drought ends and water resources increase, infected mosquitoes and birds disperse from the hammocks and initiate the early transmission phase of the Florida SLEV cycle.

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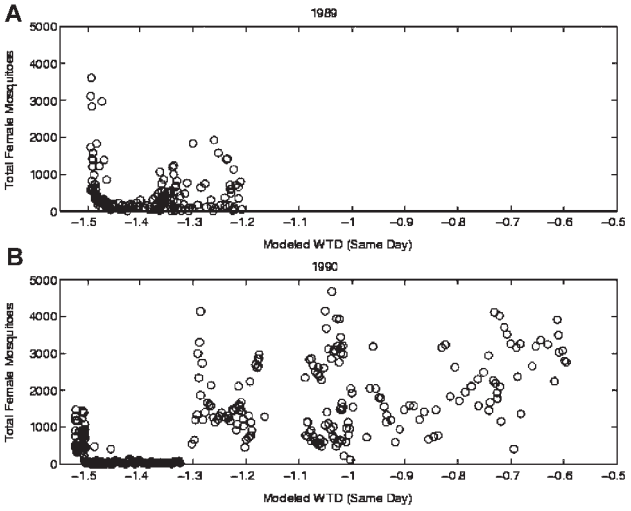

Figure 1. The 1989 and 1990 distributions of daily collected female *Culex nigripalpus* plotted as a function of water table depth (WTD), same day. A) The 1989 distribution; B) the 1990 distribution.

We expand on the approach presented in Shaman et al. (6) and further examine the relationship between modeled WTD and SLEV transmission to sentinel chickens in Indian River County, Florida. We define three types of SLEV transmission (incidence, transmission number, and epidemic transmission) and explore the relationship between these categories and modeled WTD for a longer period of record. We then develop a forecast for epidemic SLEV transmission and demonstrate the skill of this forecast. Lastly, we present a real-time forecast of epidemic SLEV transmission for the transmission season of 2002.

Modeling Overview and Methods

Topographically Based Hydrology Model

Hydrologic modeling follows the methods set forth in Shaman et al. (6). See online Appendix A for details (available from http://www.cdc.gov/ncidod/EID/vol10no5/03-0246_appa.htm). The hydrology model was run from January 1949 through June 2002 and provided a daily time series of mean WTD for the study area. Model validation was conducted by using groundwater well measurements and surface (canal) water levels provided by the St. John's Water Management District and U.S. Geological Survey (USGS) sources. Partitioning of runoff and evapo-transpiration matched bulk estimates derived from USGS sources (11). See Shaman et al. (12) for a complete description of this validation.

Sentinel Chicken Data

Changes in the annual timing and distribution of SLEV transmission to sentinel chickens have been strongly correlated with SLEV disease in humans (1). We used data from 15 different sentinel flocks, posted in Indian River County from 1978 to 2002 and maintained by personnel from the Indian River Mosquito Control District. For any given year, a maximum of eight flocks were in operation for 5 to 12 months of the year. At each site, four to six sentinel chickens were posted. Figure 2 provides a map of the region of study and flock locations.

Generally, a 1.0-mL blood sample was drawn weekly from each bird during peak transmission periods (July through November), and twice a month during the rest of the year. Blood samples were assayed for HI antibodies to SLEV at the Florida Department of Health and Rehabilitative Services, Tampa Branch Laboratory. Individual chickens that tested positive for HI antibodies were replaced with fresh ones, and the entire flock was replaced each spring.

Categories of SLEV Transmission

We define three categories of sentinel chicken seroconversion as transmission incidence, epidemic transmission, and transmission number. In a manner consistent with Shaman et al. (6), we define SLEV transmission incidence as the occurrence of seroconversion among any of the chickens at any site. Each week is treated as a separate measurement, and transmission incidence is a categoric measure: one, if one or more chickens were seropositive; or zero, if no chickens were seropositive.

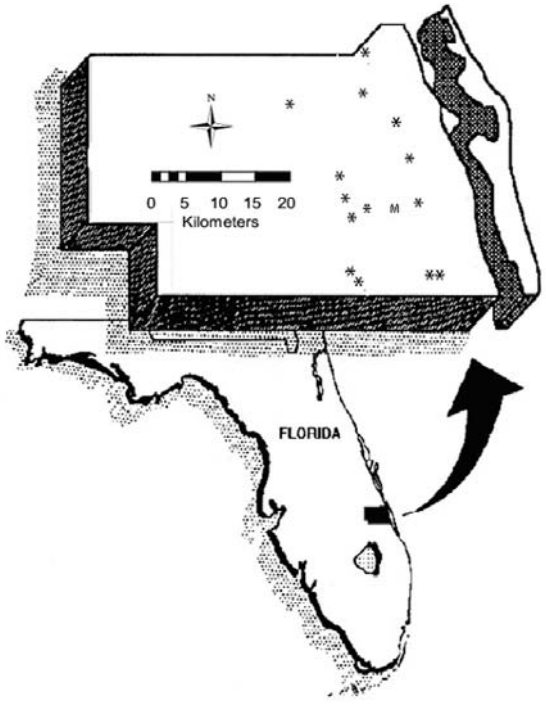

Figure 2. Map of Indian River County. Asterisks indicate the locations of sentinel chicken flocks. "M" is the site of the Vero Beach 4W meteorologic station.

High rates of sentinel chicken seroconversion are of principal interest to public health workers. These high levels of transmission connote the existence of large numbers of SLEV-infected mosquitoes and so identify times when humans are at greatest risk. We therefore define this category as epidemic transmission, which uses all the sentinel chickens in aggregate. It, too, is a categoric measure: one if ≥20% of posted chickens are seropositive in a given week; zero if ≤20% of the sentinel chickens are seropositive. These two categories represent epidemic level transmission and all other states, respectively (13).

Transmission number treats each chicken as a separate measure of SLEV transmission. Thus, for each chicken and week, the transmission number provides a categoric metric: one if the chicken is seropositive; zero if it is seronegative. For a given week, up to 48 such categoric measures are available. Unlike transmission incidence and epidemic transmission, which give a single weekly categoric value, the transmission number category provides multiple categoric measures at each point in time. These multiple measures are not necessarily independent. See online Appendix B (available from http://www.cdc.gov/ncidod/EID/vol10no5/03-0246_appb.htm) for a description of the methods used to account for this dependence.

Empiric Methods

All three types of SLEV transmission (transmission incidence, transmission number, and epidemic transmission) were defined as categoric variables. Univariate and bivariate logistic regression were used to associate the probability of each of the types of SLEV transmission with single weekly lags of modeled WTD and combinations of two lags of WTD. We defined antecedent as the longer lag and near coincident as the shorter lag. To account for the apparent dependence among chickens in the transmission number category, we performed these logistic regressions using generalized estimating equations with a working correlation ranging from r = 0–0.6, following the methods of Liang and Zeger (14). Dummy variables were also included to account for the 15 sentinel flock sites.

Logistic regression of an SLEV transmission category on modeled WTD derives the probability that this type of SLEV transmission will occur:

$$P = (1+\exp(a+ b\text{x}WTD))^{-1} \quad (1)$$

where P is the probability of SLEV transmission for a given WTD, and a and b are model parameters. See Appendix C online (available from http://www.cdc.gov/ncidod/EID/vol10no5/03-0246_appc.htm) for further description of the empiric methods.

Assessment of Forecast Skill

The quality of a forecast can be measured formally through an assessment of its skill. Skill refers to the accuracy of a forecast or set of forecasts relative to a standard control forecast. In weather and climate forecasts, often the control forecast is based on historic conditions. These historic conditions constitute a climatology, that is, a distribution of possible states. In this study our climatology is the documented frequency of epidemic SLEV transmission for each week of the year, and it is derived from the 1978 to 1997 sentinel chicken record. For each week of the 1978–1997 record, "epidemic transmission," "no epidemic transmission," or "no data" was recorded. The percentage of epidemic transmission that occurred in a given week, for instance, the 28th week of the years 1978–1997, provides the climatologic probability of epidemic SLEV transmission for that week. The climatology is fixed; hence, for each year of a forecast period, the same weekly climatologic values are predicted.

We evaluate the skill of our retrospective forecasts of epidemic SLEV transmission using the Brier score (15). The Brier score is designed for use with a probabilistic forecast of a dichotomous predictand (i.e., epidemic SLEV transmission occurred), and is calculated as follows:

$$fBS = \frac{1}{n}\sum_{k=1}^{n}(F_k - O_k)^2 \quad (2)$$

where fBS is the forecast Brier score, F_k is the forecast probability of epidemic SLEV transmission as predicted for week k, O_k is the observation of whether epidemic SLEV transmission took place during week k (O_k = 1 if epidemic transmission occurred; O_k = 0 if epidemic transmission did not occur), and n is the number of forecasts. Similarly,

$$cBS = \frac{1}{n}\sum_{k=1}^{n}(C_k - O_k)^2 \quad (3)$$

where cBS is the climatologic Brier score, and C_k is the climatologic probability of epidemic SLEV transmission as predicted for week k.

The skill score (SS) is computed directly from the Brier scores.

$$SS = 1 - \frac{fBS}{cBS} \quad (4)$$

A skill score of 0 represents no improvement of forecast skill relative to climatology. A skill score greater than 0 demonstrates improvement of the forecast relative to climatology; a skill score of 1 is a perfect forecast.

Significance of the skill score value was assessed by using a Monte Carlo procedure. The null hypothesis is that the forecasts have no greater skill than modeled WTD climatology. No skill forecasts were simulated by randomly

selecting weekly modeled WTD values from the 1949–1997 simulation record. One thousand such forecasts were made, and a mock SS was calculated for each. From this distribution of mock SS values, significance of the actual SS value was determined.

Results

Empiric Analysis

Figure 3 presents the 1978–1997 time series of weekly modeled WTD and the weekly percentage of posted chickens testing seropositive for HI antibodies. As was shown previously for 1986–1991 (6), SLEV transmission tends to occur during times of high modeled WTD after periods of low modeled WTD.

The Table presents the best-fit results, including parameter estimates, significance, and whole model goodness-of-fit for each transmission type and each time period. The relationship for 1986–1991 transmission incidence has been presented before (6). The best-fit results from analysis of the 1986–1991 record all conform to the same pattern. Antecedent drought followed by wetting favors transmission incidence, transmission number, and epidemic transmission ($p < 0.0001$, $p < 0.0001$, and $p < 0.001$, respectively). A range of values produces statistically significant logistic regression fits, reflecting the high autocorrelation of modeled WTD, i.e., wetness conditions tend to persist. For transmission number, the regression model was significant over the full range of working correlation values (r = 0–0.6). The best-fit model of transmission number is shown for a working correlation of r = 0.3.

Probabilities predicted with the 1986–1991 logistic regression model equation for epidemic SLEV transmission range from 0 to nearly 1 when combined with realistic modeled WTD scenarios (Figure 4A). These high probabilities are a consequence of the short record, which is centered upon an epidemic that began in Indian River County. Few other factors contributed to transmission during this period, and consequently there is little noise in the record.

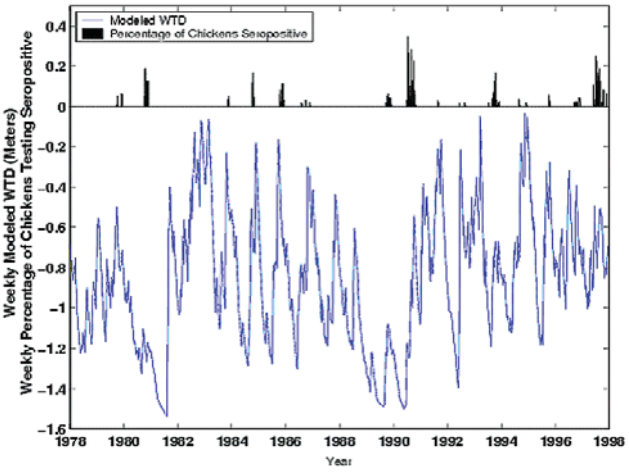

Figure 3. Time series of study data 1978–1997. The blue line is weekly modeled water table depth (WTD); the black bars are the weekly percentages of posted sentinel chickens in Indian River County testing positive for hemagglutination inhibition antibodies to St. Louis encephalitis virus.

The best-fit results from analysis of the longer 1978–1997 model show a slightly different picture. Again, for transmission incidence, antecedent drought and near coincident wetting contribute significantly to whole model goodness-of-fit ($p < 0.0001$). However, only antecedent drought (best fit 16 weeks) is significantly associated with epidemic SLEV transmission. Near coincident wetting is no longer a statistically significant explanatory variable. For transmission number, both antecedent drought and near coincident wetting contribute significantly over a range of antecedent drought lags (5–13 weeks), near coincident wetting lags (0–3 weeks), and working correlation values (r = 0–0.3). Near coincident wetting is not significant at higher working correlation values (r = 0.4–0.6). We have shown the best-fit model for a working correlation of r = 0.3.

Autocorrelation among empiric model residuals was nominal for all but the 1978–1997 transmission incidence model. For instance, weekly autocorrelation for the

Table. Best-fit empiric relationships based on logistic regression analyses between lags of modeled WTD as simulated by the topographically based hydrology model and three categories of SLEV transmission[a,b]

Predictand	1986–1991 Transmission incidence	1986–1991 Transmission no.	1986–1991 Epidemic transmission	1978–1997 Transmission incidence	1978–1997 Transmission no.	1978–1997 Epidemic transmission
Antecedent lag	17	14	11	16	8	16
Near coincident lag	2	0	0	2	2	-
Intercept	19.03 (3.74)	17.50 (1.79)	20.98 (7.07)	2.48 (0.39)	6.33 (0.46)	14.29 (3.50)
Antecedent slope	18.06 (3.65)	14.36 (1.45)	19.56 (7.03)	1.80 (0.36)	2.59 (0.38)	8.13 (2.50)
p value	<0.0001	<0.0001	<0.01	<0.0001	<0.0001	<0.005
Near coincident slope	–6.21 (1.77)	–5.51 (0.79)	–8.26 (3.85)	–0.70 (0.34)	–0.53 (0.27)	-
p value	<0.0001	<0.0001	<0.05	< 0.05	<0.05	NS
Whole model fit (p value)	<0.0001	<0.0001	<0.001	<0.0001	<0.0001	<0.001

[a]Estimates of standard error are given in parentheses. For the transmission number category, the working correlation is r = 0.3.
[b]WTD, water table depth; SLEV, St. Louis encephalitis virus; NS, not significant.

1978–1997 epidemic SLEV transmission time series drops to r = 0.42 at lag one and doesn't fall to zero until week 13. However, among the residuals of the 1978–1997 epidemic SLEV transmission regression model, the autocorrelation drops to r = 0.07 at lag one and remains closer to zero for longer lags. These findings suggest that much of the autocorrelation of the 1986–1991 transmission incidence, 1986–1991 epidemic transmission, and 1978–1997 epidemic transmission is explained by modeled WTD. However, additional factors are needed to explain 1978–1997 transmission incidence.

Figure 4B shows that the probabilities predicted with the 1978–1997 logistic regression model equation for epidemic SLEV transmission range from 0 to 0.2. Thus, deep drought does not guarantee epidemic SLEV transmission, but instead foretells an increased likelihood of such events. In this longer period of record, other factors, such as avian host susceptibility and host and vector mobility, add noise to the system and complicate the prediction of epidemic SLEV transmission. Still, deep drought does provide a probabilistic predictive measure of the chance of epidemic SLEV transmission. This empiric relationship also has a 16-week lead; we therefore can use this logistic regression model to produce a seasonal forecast.

Epidemic SLEV Transmission Forecast, Indian River County, Florida

We applied the empirical relationship established for epidemic SLEV transmission (1978–1997) to TBH model simulations of WTD for September 1997–March 2002 and produced weekly retrospective forecasts of epidemic SLEV transmission for January 1998 through June 2002. That is, we combined weekly, modeled WTD with the equation

$$P(SLEV+) = (1+\exp(14.29 + 8.13*WTD_{16}))^{-1} \quad (5)$$

where $P(SLEV+)$ is the probability of epidemic SLEV transmission, and WTD_{16} is WTD 16 weeks before. Together, the TBH simulation of WTD and equation 2 provide a weekly probabilistic forecast of the likelihood of epidemic SLEV transmission.

Figure 5 presents this time series of weekly, retrospective epidemic SLEV transmission forecast probabilities, shown in conjunction with averaged historic conditions, i.e., the climatology. For most of 1998 through 2002, our retrospective forecast predicts a lower probability of SLEV transmission than would be anticipated from historic conditions. Only during 2000 did forecast probabilities noticeably exceed those of climatology. During January 1998 through June 2002, no epidemic SLEV transmission was recorded in Indian River County.

The Brier skill score was calculated for the weekly January 1998–June 2002 retrospective forecast of epidemic SLEV transmission. A high level of skill is found (SS = 0.461) and is significant ($p < 0.001$).

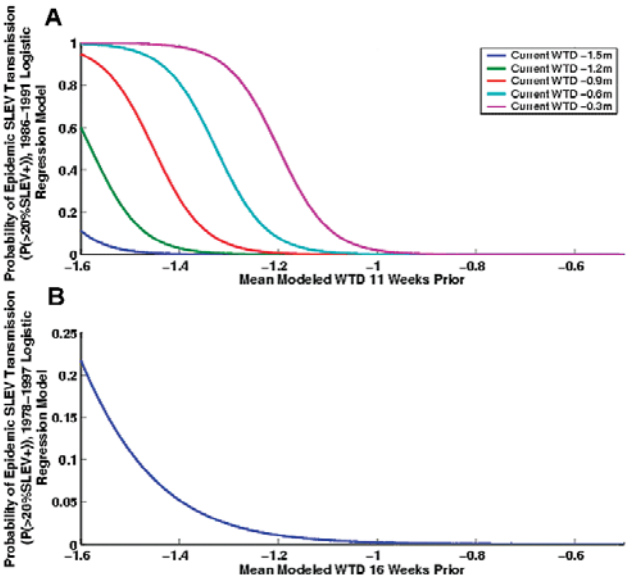

Figure 4. A) Best-fit bivariate logistic regression model of epidemic St. Louis encephalitis virus (SLEV) transmission based on the 1986–1991 record. Plotted for a continuous range of modeled water table depths (WTD) 11 weeks before transmission and fixed values of current modeled water table depths. B) Best-fit logistic regression model of epidemic SLEV transmission based on the 1978–1997 sentinel chicken record. Only antecedent drought conditions are statistically significant. Plotted for a continuous range of modeled water table depths 16 weeks before transmission.

Real-Time Forecast

Having found a high level of skill for our epidemic SLEV transmission forecast, we then developed a real-time forecast of epidemic SLEV transmission in Indian River County during 2002. (This forecast was in real time when this manuscript was prepared and initially submitted.) Model simulations from March to June 2002 were combined with equation 2 and are presented in Figure 6. The probability of epidemic SLEV transmission was predicted to be low (<2%), less than would be expected from climatology. This real-time forecast was accurate; during the fall of 2002, no sentinel chicken SLEV seroconversions occurred in Indian River County.

Discussion

In this study, we have defined three types of SLEV transmission as measured by sentinel chickens: transmission incidence, transmission number, and epidemic transmission. All three categories of SLEV transmission were found to be empirically associated with modeled WTD as simulated with the TBH model. For the shorter record, 1986–1991, antecedent drought and near coincident wetting

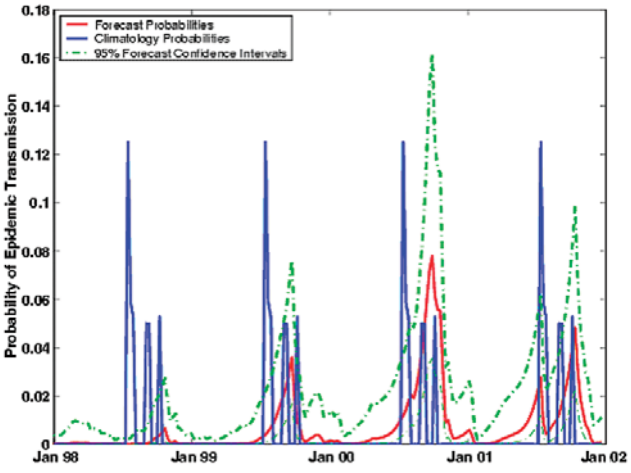

Figure 5. Time series of weekly, retrospective epidemic St. Louis encephalitis virus (SLEV) transmission forecast probabilities, January 1998–June 2002, with 95% confidence intervals. Also shown are the weekly, epidemic SLEV transmission probabilities as would be predicted from climatologic features (1978–1997).

were shown to be good predictors of all three transmission categories. These results, combined with the *Cx. nigripalpus* collection data (Figure 1), support the hypothesis of drought-induced amplification previously described (6).

The longer 1978–1997 record accounts for a wider variety of modeled wetness conditions and encompasses a longer, noisier record of sentinel chicken data, in which confounding factors, such as avian host susceptibility and host and vector mobility, may be strongly affecting transmission levels. Consequently, while not providing the same mechanistic insight into amplification dynamics as the shorter 1986–1991 record, empiric relationships derived from the 1978–1997 record provide a more realistic prediction of SLEV transmission based on modeled WTD.

For the longer 1978–1997 record, antecedent drought and near coincident wetting were significant predictors of transmission incidence. For transmission number, both antecedent drought and near coincident wetting were also significant predictors, but only for lower working correlation values (r = 0–0.3). Antecedent drought by itself, however, was a significant predictor of transmission number over the full range of working correlation values (data not shown). Lastly, logistic regression analysis of the longer record showed that only antecedent drought was significantly associated with epidemic transmission. Furthermore, for all three categories of SLEV transmission for 1978 to 1997, probabilities predicted using the logistic regression models and realistic simulated WTDs were considerably lower than those for the shorter 1986–1991 record. This finding corroborates the assertion that factors other than surface wetness conditions also control SLEV transmission rates. Consequently, drought-induced amplification may be necessary for high levels of SLEV transmission, but it alone is not a sufficient condition for such an event, nor must it occur locally.

The loss of significance for near-coincident wetting might have several causes. Epidemic SLEV transmission is a rare event. For the 1978 to 1997 Indian River County record, epidemic SLEV transmission only took place during the 1990 epidemic and on one occasion in 1997. These 20 years include many wet events, but these events were often not preceded by the drought needed for amplification of SLEV and therefore were not associated with transmission. Other factors could also have been at play, such as avian immunity, mosquito migration, human activity, and land use changes, which might have countered the effect of wetting and reduced its association with epidemic SLEV transmission to levels below significance. However, recent analysis of human cases of SLE suggests that with a still larger record, wetting would again be significantly associated with epidemic SLEV transmission (16).

Epidemic SLEV transmission, associated with human SLE incidence, is of principal epidemiologic concern. We have presented a forecast of epidemic SLEV transmission, as measured with sentinel chickens, using the empiric association between 1978–1997 epidemic SLEV transmission and modeled WTD and additional simulations of WTD with the topographically based hydrology model. Forecast skill has been demonstrated, and a real-time forecast presented. Because of wet conditions for the winter of 2001–2002 in Indian River County, springtime drought (lowering of WTD) was less severe than usual. As a result, probabilities of epidemic SLEV transmission were predicted to be lower than expected, based on climatology, for the

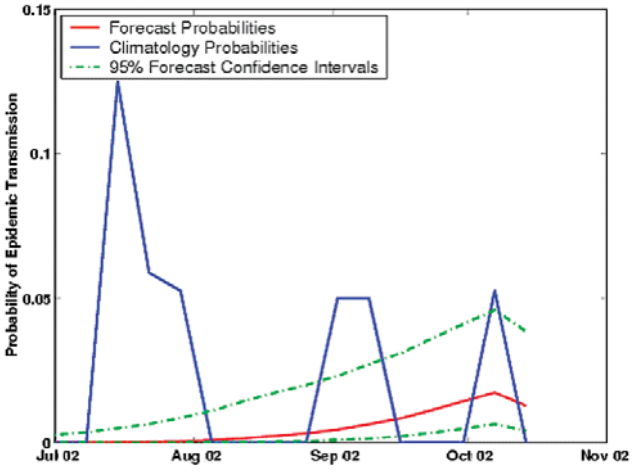

Figure 6. Real-time forecast of the probability of epidemic St. Louis encephalitis virus transmission in Indian River County, Florida, July–October 2002, with 95% confidence intervals. Also shown are the weekly climatologic probabilities of epidemic St. Louis encephalitis virus transmission.

July through October 2002 season. To our knowledge, these analyses are the first application of forecast verification methods to a predictive disease transmission model. This work demonstrates a means by which other empiric models of disease transmission can be tested for predictive skill.

We have shown that the TBH model can be used to predict SLEV transmission. Other hydrology models might also be developed and their simulations compared with those of the topographically based hydrology model. Such models would have to capture the spatial and temporal variability of near surface wetness conditions and be easily calibrated and computationally efficient. The TBH model was calibrated for 1983 to 2001 (12), but before this period, changes to the Florida landscape may have occurred. These changes, including increased channelization and urbanization, could be corrupting model simulation accuracy before 1983, and will need to be explored in more detail in the future.

A forecast of SLEV transmission should incorporate additional information regarding the dynamics of the avian hosts, mosquito vector, and virus. For instance, monitoring avian host susceptibility to the SLEV, in addition to modeling local hydrology, is needed to determine whether conditions ideal for amplification exist. Remote sensing data should also be incorporated to delineate the effects of changes in land use, urbanization, and habitat fragmentation. Future investigations might also characterize the direct effects of large-scale climate phenomena, such as the El Niño-Southern Oscillation or North Atlantic Oscillation, on SLEV transmission. Such information would help further constrain epidemic SLEV transmission forecasts and permit more accurate identification and prediction of local amplification "hot spots" throughout south-central Florida. Such forecasts could be run operationally at the state and county level in conjunction with water management and public health agencies. Forecasts of epidemic SLEV transmission in excess of climatology would then warrant response and the targeting of mosquitoes during the amplification phase.

Whether other, past south Florida SLEV epidemics conformed to similar amplification dynamics, epicenters for these epidemics must be identified, and the local hydrology modeled to determine whether a similarly timed drought and wetting pattern preceded SLEV transmission. The findings of such studies will no doubt modify the empirical relationship between modeled local hydrologic conditions and epidemic SLEV transmission (equation 2). Research is also under way to determine whether West Nile virus transmission is similarly affected by hydrologic variability. By accounting for the interaction of the physical (climate) and biologic (vector, pathogen, and host) systems, a more robust means of monitoring and forecasting disease should be attained.

Acknowledgments

We thank D. Lohmann and B. Cosgrove for provision of the Global Energy and Water Cycle Experiment Continental Scale International Project Land Data Assimilation System forcing datasets; Lillian Stark and her staff at the Florida Department of Health Tampa Branch Laboratory for their analysis of sentinel chicken and wild bird serum samples; and P. Shaman for many helpful statistical discussions.

This research was supported by the National Aeronautics and Space Administration Headquarters under the Earth System Science Fellowship, Grant NGT5-50323, the National Oceanic and Atmospheric Administration Postdoctoral Program in Climate and Global Change, administered by the University Corporation for Atmospheric Research, and the National Aeronautics and Space Administration Seasonal-to-Interannual Prediction Project at Goddard Space Flight Center.

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Acute Tick-borne Rickettsiosis Caused by *Rickettsia heilongjiangensis* in Russian Far East

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An acute tick-borne rickettsiosis caused by *Rickettsia heilongjiangensis* was diagnosed in 13 patients from the Russian Far East in 2002. We amplified and sequenced four portions of three rickettsial genes from the patients' skin biopsy results and blood samples and showed that the amplified rickettsial genes belong to *R. heilongjiangensis*, which was recently isolated from *Dermacentor sylvarum* ticks in nearby regions of China. This rickettsia, belonging to subgroup of *R. japonica*, was previously suggested to be pathogenic for humans on the basis of serologic findings. We tested serum samples with different rickettsial antigens from 11 patients and confirmed increasing titers of immunoglobulin (Ig) G and IgM to spotted fever group rickettsiae, including *R. heilongjiangensis*. Clinical and epidemiologic data on these patients show that this disease is similar to other tick-borne rickettsioses.

Russian Far East is a geographic, economic, and political unit within the Russian Federation. It consists of the smaller administrative areas (regions) located on or close to the Asian Pacific coast. The southern portion of Khabarovsk region, where this study was carried out, is situated alongside the Amur River down to the sea (Figure 1) and is characterized by peculiar combinations of subtropical and boreal biologic niches. Local experience suggests that tick-borne encephalitis, Siberian tick typhus, and, more recently, Lyme disease are prevalent in this territory, with marked seasonal disease peaks (1). In 2002, serologic evidence for acute granulocytic ehrlichiosis was found in the region (2).

In 1932, a disease described as tick-borne fever (tick typhus) was identified in the Russian Far East (3). In Central Siberia in 1935, the agent of tick-borne fever, *Rickettsia sibirica*, was isolated and described (4–6), and several rickettsial isolates from the ticks from the Far Eastern Russia have also been identified as this new species (7). Since 1935, the cause of acute tick-borne spotted fever in the Russian Far East (including Khabarovsk Region) is thought to be *R. sibirica*, and the antigen of *R. sibirica* has been used for serologic studies in clinical laboratories. Nevertheless, differences between clinical pictures of tick-borne fever and differences in serologic cross-reactivity of sera from patients with tick-borne rickettsiosis have been reported in Siberia and the Russian Far East (8). *Dermacentor sylvarum* ticks have been identified as a vector. In 1990s, several strains of *R. sibirica* were also isolated from ticks in Primorye Region, south of Khabarovsk Region (9).

In Siberia and China, several new rickettsiae were found. *R. mongolotimonae* found in Inner Mongolia is closely related to *R. sibirica* (10) and causes acute disease in humans. Clinical cases have now been described in France (11,12). *R. heilongjiangensis* (strain 054) was first isolated from *D. sylvarum* ticks collected around Suifenhe in the Heilongjiang Province of China in 1982 (13). Serologic studies indicated that the organism was the probable cause of an acute tick-borne disease in humans (14). *R. hulinensis* (strain HL-93) was isolated in 1993 from *Haemaphysalis concinna* ticks collected in Hulin County, Heilongjiang Province (10). The pathogenicity of this agent in humans is unknown. By using molecular evolutionary genetic analyses, these rickettsiae were shown to form a well-defined clade distinct from other spotted fever group rickettsiae, including *R. japonica* (15). Both *D. sylvarum* and *H. concinna* ticks inhabit the Russian Far East, although *D. sylvarum* is quite rare (1). In this study, we identify the cause of an acute, febrile, tick-transmitted disease in the Russian Far East and describe the clinical picture in these cases.

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Figure 1. Geographic location of study area.

Patients and Methods

Sample Collection

During this study in 2002, we screened almost all patients who were suspected clinically to have a tick-borne rickettsiosis and who were hospitalized in the infectious diseases department of Khabarovsk Municipal Hospital No.10. Five to 20 days before onset of the disease, most patients reported tick bites or tick exposure. Some had typical eschars on the skin without a history of a tick bite. The illness was acute, febrile, and usually involved a macular or maculopapular rash. All patients were successfully treated with a 14-day course of doxycycline. Patients with clinically evident features of tick-borne encephalitis (neurologic signs) or Lyme disease (erythema migrans) were excluded from the study. In total, samples from 65 patients were investigated, 64 blood samples and 17 skin biopsy results. Paired sera from most of the patients were tested with conventional serologic methods. The first serum sample was taken immediately after admission to the hospital, usually 1–9 days after the onset of the disease. The second serum sample was taken 4–18 days later.

DNA Extraction, PCR, and Sequencing

Buffy coats were separated from fresh blood specimens immediately after collection at patient's admission. DNA was extracted from the buffy coat and skin biopsies samples with QIAmp DNA Blood Mini Kit and DNeasy Tissue Kit (Qiagen, Tokyo, Japan) according to manufacturer's instructions.

Oligonucleotide primers used in this study and annealing temperatures are shown in Table 1. We used the nested polymerase chain reaction (PCR) method for primary detection of the rickettsial DNA in human samples. The *gltA* (citrate synthase) gene was chosen as the target for amplification because of its genus specificity and conservativeness. The primer CS1d (16), which is used traditionally to amplify the full-length *gltA* gene, is complementary to the ultimate 5′-end of the open reading frame for this gene of *R. prowazekii*. Since we expected to find DNA of the spotted fever group rickettsiae, we modified the primer to be completely homologous to the corresponding portion of the gene of *R. conorii* (Table 1). *R. conorii* has been chosen as a template to design primer CS2d as the only spotted fever group rickettsia having a completely sequenced genome at the time of investigation. Comparison of these two primers indicated that the newly designed primer (CS2d) was at least 100 times more sensitive than CS1d in the amplification of the serially diluted DNA of *R. montanensis*, which was used as a positive control (data not shown). Primers CS2d and CSendR amplify the full-length of the *gltA* gene. Primers CS877f and CS1258r were used in the nested PCR assay. To amplify the full-length of the *gltA* gene, we used PCR followed by hemi-nested PCR using the product of the first PCR as a template. Two conservative regions of the outer membrane protein A (*ompA*) gene were amplified (base positions 91–680 and 3,608–6,789 with respect to the sequence published for *R. rickettsii*) by using previously described primers (17). Initial amplification of the 3,182-bp longer region was achieved with primers 190-3588f and 190-5044r and with primers 190-4859f and 190-6808r. Products of this reaction were used later in the nested and hemi-nested PCR reactions with corresponding primers. The *ompB* gene was amplified from clinical samples and DNA by using standard primers (18), except for the ultimate forward primer 120-M59, which was found to produce nonspecific amplicons with the human DNA in clinical samples. Instead, for clinical specimens, we used a newly designed primer, 120-M36. As with the *ompA*, the DNA from clinical samples was amplified in two steps. In the first step, we amplified two halves of the gene, with a small overlapping region with primers 120-M36 with 120-2988 and 120-2788 with 120-4879. The products of these reactions were used as templates for each of the specific nested or heminested reactions for seven regions. All

Table 1. List of the primers used to detect rickettsial DNA

Gene for amplification	Primer name	Primer sequence 5′-3′[a]	Annealing temperature
gltA	CS1d	ATGAC**T**AATG**GC**AATAATAA	
	CS2d	ATGAC**C**AATG**AA**AATAATAA**T**	50°C
	CSEndr	CTTATACTCTCTATGTACA	
	RpCS877p	GGGGACCTGCTCACGGCGG	54°C
	RpCS1258n	ATTGCAAAAAGTACAGTGAACA	
ompA, 5′- portion	190-70	ATGGCGAATATTTCTCCAAAA	53°C
	190-701	GTTCCGTTAATGGCAGCATCT	
ompA, 3′- portion	190-3588f	AACAGTGAATGTAGGAGCAG	46°C for the first round 50°C for the nested and heminested rounds
	190-3968r	TAGCAGCTGATTTAGTAGCT	
	190-4084f	CATCACCGATATTTCTAGC	
	190-4338f	TTCAGGAAACGACCGTACG	
	190-4406r	ACTATACCCTCATCGTCATT	
	190-4859f	GCGAAATCCAAGGTACAGG	
	190-5044r	AACTTGTAGCACCTGCCGT	
	190-5125f	GCGGTTACTTTAGCCAAAGG	
	190-5238r	ACTATTAAAGGCTAGGCTATT	
	190-5768f	CACCGCTACAGGAAGCAGAT	
	190-5831r	GTGTCGCTAGGTTTTACAAC	
	190-6228f	CGTTGAAGTATAGCATC	
	190-6427r	ATCTAAGCCCAGCTAGCGGT	
	190-6808r	CACGAACTTTCACACTACC	
ompB	120-M36	TTCTACAGCTACCATAGTAGCCA	50°C
	120-607	AATATCGGTGACGGTCAAGG	
	120-807	CCTTTTAGATTACCGCCTAA	
	120-1378	TAAACTTGCTGACGGTACAG	
	120-1497	CCTATATCGCCGGTAATT	
	120-2113	CGATGCTAACGTAGGTTCTT	
	120-2339	CTTGTTTGTTTAATGTTACGGT	
	120-2778	AAACAATAATCAAGGTACTGT	
	120-2988	CCGGCTATACCGCCTGTAGT	
	120-3462	CCACAGGAACTACAACCATT	
	120-3599	TACTTCCGGTTACAGCAAAGT	
	120-4232	GGTTTCTCATTCTCTCTATATGG	
	120-4346	CGAAGAAGTAACGCTGACTT	
	120-4879	TTAGAAGTTTACACGGACTTTT	

[a]Differences between primers CS1d and CS2d are indicated by bold letters.

primers were purchased from Eurogentec (4102 Seraing, Belgium).

Amplification has been carried out by routine methods in a final volume of 50 μL with appropriate negative controls (10,17). In all reactions, as a positive control, we used a mixture of DNA of the following microorganisms: *Anaplasma phagocytophilum*, *Neorickettsia sennetsu*, *Wolbachia pipientis*, *Francisella tularensis*, *Bartonella henselae*, *Borrelia garinii*, *Coxiella burnetii*, and *R. montanensis*. We found that the cocktail of DNA of tick-borne or phylogenetically close to tick-borne bacteria is convenient to use in PCR with potentially polyinfected human samples.

We checked all samples for other possible tick-borne bacterial and pathogens with the following primers under conditions suggested in published references: SL primers for *ospA* gene of *Borrelia* (19); BhCS.781p and BhCS.1137n primers for *Bartonella* citrate synthase (20); HE1, HE3, and HE4 primers for 16S rDNA of *Ehrlichia chaffeensis* (21); GE3A, GE10r, GE9f, and GE2 primers for 16S rRNA gene of *A. phagocytophilum* (22); and P3708 and p4257 primers for the gene encoding p44 protein *of A. phagocytophilum* (23) [data not shown]. No positive results have been obtained among patients described here.

The PCR products were purified for DNA sequencing with the QIAquick PCR purification kit (Qiagen) and then directly sequenced by using PCR primers. Sequencing reactions were carried out with a D-rhodamine terminator cycle DNA sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequencing reaction products were resolved by electrophoresis with an ABI Prism 377 sequencer (Applied Biosystems). The results obtained were processed into sequence data with AutoAssembler software (Applied Biosystems). The sequences of the *gltA*, both regions of the *ompA* gene, and the *ompB* genes were aligned by using the software Genetix-Win 5.1 (Software Development Co., Ltd., Japan). Sequences in the *ompB* genes of *R. heilongjiangensis* and *R. hulinensis* were not available in the

GenBank database so we amplified and sequenced the gene. The sequences used for comparison were obtained from the GenBank database, aligned, and then corrected manually to preserve codon alignment and conserved motifs. Sites with ambiguous alignments were removed before phylogenetic analysis. The phylogenetic tree was calculated by using neighbor-joining method with MEGA2 Version 2.1 software (available from http://megasoftware.net). Internal node support was verified by using the bootstrap method with 100 replicates.

Serologic Studies

Two serologic tests were performed in Khabarovsk Plague Control Station: immunofluorescence studies with a combined antigen consisted of two local strains of *B. garinii* and one local strain of *B. afzelii*, and an enzyme-linked immunosorbent assay detected antibodies against tick-borne encephalitis virus (24). For further investigations, sera were dried on blotting papers as described previously (25) and transported to Marseilles, where microimmunofluorescence testing (26) was performed by using in-house prepared antigens of *R. heilongjiangensis* (strain 054, ATCC VR-1524), *R. hulinensis* (strain HL-93, ATCC VR-1527), *R. sibirica* (strain 246, ATCC VR-151), *R. conorii* (strain Moroccan ATCC VR-141), *C. burnetii* (strain Nine Mile, ATCC VR-615), *Orientia tsutsugamushi* (strains Gilliam, Karp, Kato, and Kawazaki), *E. chaffeensis* (strain Arkansas), *A. phagocytophilum* (strain Webster), *Bartonella henselae* (Houston-1, ATCC-49882), and *B. quintana* (strain Oklahoma). Antigens were applied by pen point to 18-well microscope slides, dried for 30 min, and fixed. Appropriate positive- and negative-control serum samples were tested on each slide together with twofold dilutions of patients' serum samples made in 3% nonfat dry milk in phosphate-buffered saline (PBS). Slides were incubated in a moist chamber for 30 min at 37°C, washed twice in PBS and once in distilled water (10 min each); reactive antibodies showed fluorescein isothiocyanate–conjugated goat anti-human γ chain and μ-chain immunoglobulins (BioMérieux, Marcy l'Etoile, France). After the conjugate was added, slides were incubated for 30 min at 37°C, washed in two PBS for 10 min and for 5 min in distilled water, and mounted in buffered glycerol. Endpoints for each antigen were the lowest concentrations of serum that definitely conferred fluorescence on bacteria.

Nucleotides Accession Numbers

Nucleotide sequences obtained during this study were deposited in GenBank under the following numbers: AY260451 for *ompB* gene of *R. heilongjiangensis*, strain 054; AY260452 for *ompB* gene for *R. hulinensis*; AY280712 for *ompB* gene; AY280711 for previously tandemly repeated region portion of *ompA* gene; AY280710 for another portion; and AY280709 for *gltA* gene of *Rickettsia heilongjiangensis* found in this study.

Results

We amplified and sequenced DNA of *R. heilongjiangensis* in samples from 16 patients. Serum samples from 11 were available for serologic studies, and clinical and epidemiologic data have been analyzed for 13 patients, including all patients with investigated serum samples.

Ten of 17 samples of DNA extracted from skin eschars and seven of 64 samples of DNA extracted from buffy coats were positive in the nested PCR for the *gltA* gene. In one patient, both the skin biopsy and the buffy coat were positive and had the same DNA sequence. Because we had limited amounts of extracted DNA, we attempted to amplify both the *ompA* and *ompB* genes from six samples (three skin biopsies and three blood samples), which were previously positive in nested PCR with primers for citrate synthase gene. All positive samples were also screened by PCR for other possible bacterial tick-borne pathogens and were found to be negative. Results from testing, serologic or PCR, that suggested double infection were excluded from the study. Clinical picture of the disease was analyzed in patients with *R. heilongjiangensis* infection to describe the disease associated with this organism.

All 17 nested PCR amplicons of amplified *gltA* gene were directly sequenced and showed 100% homology. Six amplicons of *ompA* and *ompB* genes of corresponding samples were also identical with each other. We obtained full-length *gltA* gene sequence, 590 bp and 3,182 bp (excluding primer sequences) of 5′- and 3′-regions of the *ompA* gene, respectively, and a 4,852-bp length sequence of the *ompB* gene. A BLAST search showed that all sequences were completely homologous to correspondent genes of *R. heilongjiangensis*. Figure 2 shows the phylogenetic relationships of this *Rickettsia* and other species based on the analysis of both concatenated portions of the *ompA* gene.

Serum samples from 11 of 16 patients were studied; data are presented in Tables 2 and 3. None of the samples had serologic evidence of any other nonrickettsial acute, tick-borne disease. One previously vaccinated patient had a low titer of immunoglobulin (Ig) G against tick-borne encephalitis virus, and two had low titers to both *B. henselae* and *B. quintana* (data not shown). In 9 of 11 available paired sera from PCR-positive patients, we found serologic evidence for acute rickettsial infection, e.g., the presence of IgM antibodies, seroconversion, or a fourfold rise in antibody titer against rickettsial antigens. In samples from two patients, IgG and IgM antibody titers to *R. heilongjiangensis* were highest. In samples from five patients, the same titers were present against *R. conorii* and *R. heilongjiangensis*. In samples from one patient, we found the

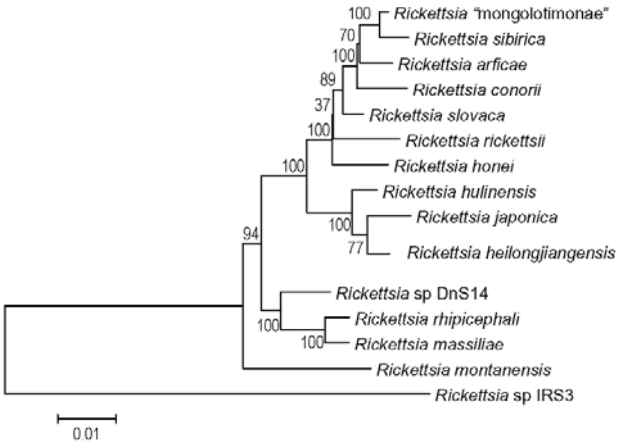

Figure 2. Phylogenetic tree, representing position of *Rickettsia heilongjiangensis*. The tree was made using MEGA 2.1 software after alignment of both portions of *ompA* genes obtained from GenBank and sequenced during this study by GENETIX-WIN 5.1 software. The scale represents a number of substitutions per base per indicated horizontal distance. The numbers present at nodes of the tree represent the number of bootstrap replicates of 100 that display the indicated sequence groupings.

same levels of antibodies to all rickettsial antigens. Levels of antibodies against *R. sibirica* in all cases, except one, were lower than against *R. heilongjiangensis.* In four of nine cases, titers were identical against antigens of *R. hulinensis* and *R. sibirica.* Absence of specific antibodies against rickettsiae in patients 2 and 3 could be explained either by early treatment with a specific antimicrobial drug or probable errors in serum sample collection and treatment.

Epidemiologic, clinical, and laboratory data available in 13 of 16 PCR-positive patients are given in Table 4. Before the onset of the disease in the summer 2002 (from June to August), all patients had a history of tick bite, tick exposure, or a stay in an epidemiologically suspected location. After an incubation period of 4 to 7 days, the patient had a sudden onset with fever, but no specific symptoms appeared during the first several days. In 12 patients, a macular or maculopapular rash appeared but was usually faint. Twelve patients had a primary lesion (eschar) at the site of tick attachment (Figure 3). The eschar consisted of a necrotic central region (50–150 mm in diameter) surrounded by infiltrated and inflamed tissue (70–400 mm in diameter), and a zone of hyperemia (250–500 mm in diameter). The eschars were found on the waist and buttocks region (four); lower extremities (two); upper extremities and axillar region (two); and back, chest, neck, and abdomen (one case each). In two patients, we noticed subcutaneous lymphangitis and regional lymphadenopathy. Initial conventional treatment at home, with a combination of antipyretics, analgesics, and antibacterial therapy (oral

Table 2. Serologic data of 11 patients with *Rickettsia* infection[a]

Patient no.	Disease onset date	Antimicrobial treatment[b]	PCR		Sera	Collection date	IgM tests			
			gltA gene	*ompA* and *ompB* genes			*R. heilongjiangensis*	*R. hulinensis*	*R. sibirica*	*R. conorii*
1	6/18	6/23	+	NI	1	6/24	0	0	0	0
					2	6/28	1/32	1/32	1/32	1/32
2	6/20	6/23	+	+	1	6/24	0	0	0	0
					2	7/9	0	0	0	0
3	6/21	6/24	+	NI	1	6/5	0	0	0	0
					2	7/12	0	0	0	0
4	6/22	6/26	+	NI	1	6/26	1/32	1/16	1/16	1/32
					2	7/1	1/64	1/64	1/64	1/64
5	6/22	6/28	+	NI	1	6/28	0	0	0	0
					2	7/16	1/128	1/32	1/32	1/128
6	6/29	7/2	+	+	1	7/3	0	0	0	0
					2	7/8	1/64	1/64	1/64	1/64
7	6/30	7/4	+	NI	1	7/5	0	0	0	0
					2	7/16	1/1,024	1/1,024	1/256	1/1,024
8	7/1	7/8	+	NI	1	7/8	0	0	0	0
					2	7/26	1/512	1/126	1/126	1/512
9	7/6	7/12	+	NI	1	7/15	1/64	1/64	1/64	1/64
					2	7/31	1/128	1/128	1/128	1/128
10	7/23	7/28	+	+	1	7/29	0	0	0	0
					2	8/13	1/32	1/32	1/16	1/16
11	7/25	8/1	+	NI	1	8/2	1/256	1/256	1/64	1/64
					2	8/15	1/256	1/256	1/64	1/64

[a]PCR, polymerase chain reaction; Ig, immunoglobulin; NI, not investigated.
[b]Date of beginning of antimicrobial drug therapy.

Table 3. Serologic data of 11 patients with *Rickettsia* infection

Disease onset date	Antimicrobial treatment[b]	PCR		Sera	Collection date	IgG tests			
		gltA gene	*ompA* and *ompB* genes			*R. heilongjiangensis*	*R. hulinensis*	*R. sibirica*	*R. conorii*
6/18	6/23	+	NI	1	6/24	0	0	0	0
				2	6/28	1/1024	1/512	1/512	1/1,024
6/20	6/23	+	+	1	6/24	0	0	0	0
				2	7/9	0	0	0	0
6/21	6/24	+	NI	1	6/5	0	0	0	0
				2	7/12	0	0	0	0
6/22	6/26	+	NI	1	6/26	1/64	1/64	1/64	1/64
				2	7/1	1/2,048	1/512	1/512	1/2048
6/22	6/28	+	NI	1	6/28	0	0	0	0
				2	7/16	1/128	1/128	1/128	1/128
6/29	7/2	+	+	1	7/3	0	0	0	0
				2	7/8	1/128	1/64	1/64	1/128
6/30	7/4	+	NI	1	7/5	0	0	0	0
				2	7/16	1/256	1/128	1/256	1/256
7/1	7/8	+	NI	1	7/8	0	0	0	0
				2	7/26	0	0	0	0
7/6	7/12	+	NI	1	7/15	1/1,024	1/1,024	1/1,024	1/1,024
				2	7/31	1/1,024	1/1,024	1/1,024	1/1,024
7/23	7/28	+	+	1	7/29	0	0	0	0
				2	8/13	1/1,024	1/1,024	1/256	1/256
7/25	8/1	+	NI	1	8/2	1/256	1/256	1/128	1/128
				2	8/15	1/256	1/256	1/128	1/128

[a]PCR, polymerase chain reaction; Ig, immunoglobulin; NI, not investigated.
[b]Date of beginning of antimicrobial drug therapy.

penicillins) did not result in improvement; 5–9 days after the onset of symptoms, all patients were admitted to the hospital. They received oral doxycycline for 14 days and antihistamine therapy, and clinical symptoms resolved within 2 to 3 days. Laboratory tests in the hospital showed elevated levels of serum transaminases (alanine aminotransferase and aspartate aminotransferase) in 46% and 15% of patients, respectively, and these remained elevated even during convalescence but were normal at follow-up at 3 to 4 weeks.

Discussion

Our findings suggest that patients had an acute infection with *R. heilongjiangensis*. Clinical data were typical for acute rickettsial infections. We successfully amplified four portions of three different rickettsial genes from human blood and skin samples, which suggested the presence of this microorganism. We did not amplify any of these genes in samples from healthy donors or from patients suffering from other infectious diseases (negative controls). The *gltA* gene is conservative, especially among *Rickettsia* species (16), and *ompA* and *ompB* genes encode main surface proteins of rickettsiae. These gene sequences were completely homologous to the spotted fever group *R. heilongjiangensis*. Previous GenBank sequences were recently corrected (Fournier PE et al., unpub. data). The identity of sequenced PCR products among patients, clinical data, and epidemiologic data suggest the focality of this rickettsiosis.

The serologic data support PCR and sequencing findings. We showed that *Rickettsia* produces a clear immunologic response in patients. We studied paired sera from patients for reactivity with antigens of several species in the *Rickettsia* genus, including *R. heilongjiangensis* and *R. hulinensis*. Seroconversion, presence of IgM, or increasing antibody titers were observed in most patients. In seven cases, sera reacted at higher titers with antigen of *R. heilongjiangensis* when compared with *R. sibirica*, the only currently identified tick-borne rickettsia in the Russian Far East. In 77%, the titers were identical against *R. heilongjiangensis* and *R. conorii* antigens. Although serologic cross-reactions are common among rickettsiae of the spotted fever group (26), the finding of lower titers against the phylogenetically more closely related *R. hulinensis* than the relatively more distant *R. conorii* was unexpected.

Epidemiologic evidence of tick bite or exposure, rash, primary lesion (eschar) at the site of tick bite, and rapid recovery after doxycycline treatment support a rickettsial cause for the disease. Some peculiarities were noticed when signs were compared with infection caused by *R. sibirica* in the Central Siberia. Seasonal peak of infections is in the end of June and July. For Siberian tick typhus, the seasonal peak is the end of April and May. The rash that accompanies tick-borne rickettsiosis in the Russian Far East is less obvious, and the disease apparently affects older people than Siberian tick typhus. Only 1 of 13 patients was >45 years of age. Generally, the disease is mild, with no serious complications or death recorded.

Table 4. Epidemiologic, clinical and laboratory data of 13 patients with rickettsiosis.

Feature or sign	Value (n = 13)
Sex, male/female	8/5
Age, y, mean	52 (18–66)
Mean period between onset and hospitalization, d	4.6
Mean stay at the hospital, d	5.7
Primary diagnosis of rickettsiosis at admission	9
History of tick bite	6
Incubation period, d, median (range)	5.5 (4–7)
Antibiotics taken before hospitalization	2
Chills	13
Malaise	13
Headache	13
Dizziness	11
Myalgias, arthralgias	13
Nausea	2
Anorexia	13
Maculopapular rash	12
Rash appearance after onset of disease, d, median	3.6
Duration of rash, d, median (range)	5.5 (4–7)
Presence of eschar	12
Lymphadenopathy regional to the eschar	10
Subcutaneous lymphangitis, leading to regional lymph nodes	2
Hepatomegaly	5
Splenomegaly	2
Sleep disturbances	7
Leukocytosis at admission, (>9,000/mm^3)	6
Leukopenia at admission, (<4,000/mm^3)	2
Increased ESR (>15 mm/h for men, >20 mm/h for women)	12
Thrombocytopenia, (<150,000/mm^3)	3
Proteinuria (≥0.033 g/L)	1
Increased ALT activity, >1.5 times	6
Increased AST activity, >1.5 times	2
Doxycycline treatment, 100 mg twice daily for 14 d	13

[a]ESR, erythrocyte sedimentation rate; ALT, alanine aminotransferase; AST, asparate aminotransferase.

The epidemiology of the disease remains mostly unknown. Recently, DNA of the *Rickettsia* described in our report was amplified from *H. concinna* ticks collected in Siberia (S. Shpynov, unpub. data).

PCR-based technologies and direct sequencing provide a fast and precise diagnosis or rickettsiosis. The preferable method may be PCR on eschar biopsy samples because this technique has high sensitivity and probability of finding rickettsial DNA (27). Serologic studies of samples from Russian Far East area should include tests with antigens of *R. heilongjiangensis*.

Results of our studies showed that acute febrile tick-borne disease caused by *R. heilongjiangensis* is prevalent in the Russian Far East. Molecular biology approaches enabled us to identify the cause of an acute disease and to detect its bacterial origin. As no evidence of *R. sibirica* human infection was found in our study, further investigations are needed clarify its role in human pathology in the Russian Far East, especially the Khabarovsk Region.

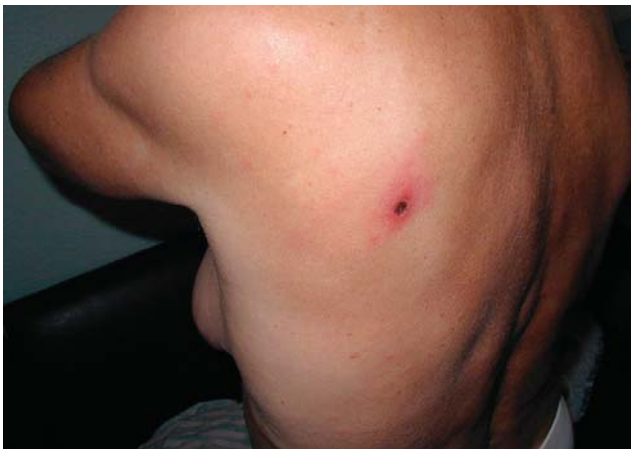

Figure 3. Eschar and faint macular rash in patient 9.

Acknowledgments

We are grateful to S. Dumler and M.M. Eremeeva for worthy advice, G.S. Tomilka and V.I. Zholondz for assistance in samples collection, and P. Kelly for thoroughly reviewing this manuscript.

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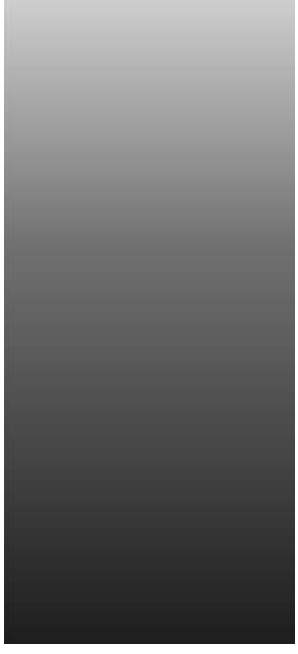

Clinical Manifestations, Laboratory Findings, and Treatment Outcomes of SARS Patients

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Clinical and laboratory data on severe acute respiratory syndrome (SARS), particularly on the temporal progression of abnormal laboratory findings, are limited. We conducted a prospective study on the clinical, radiologic, and hematologic findings of SARS patients with pneumonia, who were admitted to National Taiwan University Hospital from March 8 to June 15, 2003. Fever was the most frequent initial symptom, followed by cough, myalgia, dyspnea, and diarrhea. Twenty-four patients had various underlying diseases. Most patients had elevated C-reactive protein (CRP) levels and lymphopenia. Other common abnormal laboratory findings included leukopenia, thrombocytopenia, and elevated levels of aminotransferase, lactate dehydrogenase, and creatine kinase. These clinical and laboratory findings were exacerbated in most patients during the second week of disease. The overall case-fatality rate was 19.7%. By multivariate analysis, underlying disease and initial CRP level were predictive of death.

Severe acute respiratory syndrome (SARS) is a new infectious disease, first recognized in November 2002 (1). SARS has spread rapidly around the world: >8,400 cases have been reported from 30 countries on five continents (2,3).

Previous reports have described some major clinical findings of SARS, including the temporal progression of clinical symptoms and chest radiography, the outcomes, suggested treatment protocol, and risk factors for death (4,5). However, data are still limited on the temporal progression of abnormal laboratory findings, such as leukopenia, lymphopenia, thrombocytopenia, elevated lactate dehydrogenase (LDH), elevated aspartate aminotransferase (AST), elevated alanine aminotransferase (ALT), elevated creatine kinase (CK), and elevated C-reactive protein (CRP) and the roles each of these plays in predicting outcomes and complications. Although So et al. have suggested a treatment protocol with the emphasis on early use of high-dose steroids (6), whether this treatment is better than others is unclear.

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We conducted a prospective study on the clinical, radiologic, and hematologic findings of SARS patients with pneumonia, who were admitted to National Taiwan University Hospital (NTUH) from March 8 to June 15, 2003. Most of these patients were treated following a standard treatment protocol, different from that suggested by the study group in Hong Kong (6). We report on the clinical features of our SARS patients with pneumonia, with emphasis on temporal progression of laboratory findings, treatment outcome, and risk factors for poor prognosis.

Methods

Setting

NTUH is a 2000-bed, university-affiliated medical center located in northern Taiwan. The center provides both tertiary and primary care for patients. It was also the primary hospital caring for SARS patients during the SARS outbreak in Taiwan.

Patient Description and Treatment Protocol

All patients who fulfilled the revised World Health Organization (WHO) definition of probable SARS (7) in whom pneumonia developed and who received treatment at NTUH during March 8 to June 15, 2003, were enrolled in this study. Except for the first patient, who did not receive any of the following treatments, and the second, third, and fourth patients, who received steroid in the first week of their disease, all patients received treatments that conformed to the guideline described here. Oral ribavirin was prescribed soon after the diagnosis of SARS was made; the loading dose was 2,000 mg followed by 1,200 mg per day if the body weight was >75 kg, or 1,000 mg per day if the body weight was <75 kg. This treatment lasted 10 days unless adverse effects developed. Antimicrobial agents for community-acquired pneumonia, either moxifloxacin alone or ceftriaxone plus azithromycin, were administered at the same time. Methylprednisolone was usually administered in the second week of the disease if any of the following occurred: a flare of fever, progression

of clinical symptoms (such as dyspnea or diarrhea), a surge or resurge of CRP level, or rapid deterioration of chest radiographic findings (development of new infiltration). Methylprednisolone was indicated in the first week of disease only if clinical symptoms or laboratory abnormalities (such as elevated CK, LDH, CRP) worsened rapidly, and rapidly progressed abnormalities were found on chest radiograph. The dosage was 2 mg/kg/day for 5 days, and then it was tapered off. Pulse therapy with methylprednisolone, 500 mg/day for 3 days, was used if there was major disease progress under the standard regimen. Intravenous immunoglobulin (IVIG) was administered if severe leukopenia ($<2 \times 10^9$/L), thrombocytopenia ($<100 \times 10^9$/L), or both occurred, or if lesions on chest radiography progressed rapidly in the first week of disease. The dosage of IVIG was 1 g/kg/day for 2 days. Once patients were intubated and supported by a mechanical ventilator, respiratory care followed the principles suggested for managing acute respiratory distress syndrome (8).

Laboratory Examination

The etiologic workup included the sputum Gram stain and acid-fast stain, sputum culture for bacteria, sputum chlamydial antigen, throat swab for virus isolation, urine pneumococcal antigen, and urine legionella antigen. We tested antibody reactions of both acute- and convalescent-phase serum specimens, 4 weeks apart, for *Mycoplasma, Chlamydia* influenza virus, parainfluenza virus, adenovirus, coxsackievirus, respiratory syncytial virus, and SARS-related coronavirus (SARS-CoV). We also took throat swabs for reverse transcription–polymerase chain reaction (RT-PCR) for SARS-CoV. The other routine laboratory tests, such as the hemogram, serum AST, ALT, CK, LDH, and CRP level, were examined every other day during hospitalization. A chest radiography was also performed every other day during hospitalization.

Infection Control Measures

Once a patient was diagnosed as having SARS, he or she was sent to a negative-pressure ventilated room immediately. No visitor or family member was allowed to enter this room. All healthcare workers caring for SARS patients were asked to adhere strictly to contact and airborne precautions. Before entering isolation rooms to care for SARS patients, all healthcare workers washed their hands and put on personal protective equipment, including gowns, gloves, N95 respirators, goggles, and face shields. After caring for SARS patients, such workers were to take off the personal protective equipment in the anteroom and wash their hands before leaving the isolation room. The health of healthcare workers who had any contact with SARS patients or their environments was monitored daily for 14 days after the last exposure. Once fever developed in a worker, he or she was immediately hospitalized and placed in isolation in a specially designated ward.

Data Collection

A standard case report form modified from one designed by the Centers for Disease Control and Prevention for SARS was used to collect demographic and clinical data (9). Severity of underlying disease was classified by using the modified risk stratification proposed by McCabe: rapidly fatal (death expected within 1 year), ultimately fatal (death expected within 5 years), or nonfatal (death expected >5 years or no underlying disease) (10).

Statistics

All statistical analysis was performed with SPSS version 10.0 (SPSS, Chicago, IL). Logistic regression was used for univariate and multivariate analysis. Continuous variables were compared with the *t* test. Categorical variables were compared by using the Fisher exact test. A p value < 0.05 was considered significant.

Results

During the study period, 76 patients were enrolled. Their demographic and clinical data are detailed in Table 1. The male-to-female ratio was 34:42. Their age was 24–87 years (median 46.5 years). Twenty-four patients had various underlying diseases, including cardiovascular disorders in 13 patients, diabetes mellitus in 10, hepatobiliary disorders in 6, history of cerebrovascular accidents in 3, chronic renal diseases in 2, pulmonary fibrosis in 1, history of intravenous drug abuse in 1, and adrenal insufficiency in 1. Fourteen of these 24 patients had underlying diseases classified as rapidly fatal (diabetes mellitus, ischemic heart disease, plus congestive heart failure in four patients; diabetes mellitus, ischemic heart disease, plus cerebrovascular accident with being bedridden in three; diabetes mellitus, ischemic heart disease, plus end-stage renal disease in two; diabetes mellitus plus decompensated liver cirrhosis in one; and ischemic heart disease plus massive ischemic bowel in one) or ultimately fatal (severe pulmonary fibrosis in one, ischemic heart disease in two). Most frequent initial symptoms were fever, cough, myalgia, dyspnea, diarrhea, and rigor. Three of the 24 patients who had diarrhea had previously received various antimicrobial agents. The duration from symptom onset to a patient's visiting NTUH was 1–12 days (median 3 days). The initial laboratory data are detailed in Table 2. Abnormalities on chest radiography suggesting pneumonia were found in 56 of the 76 patients. Lesions were found in one lobe in 33 patients, two lobes in 15 patients, three lobes in 4 patients, four lobes in 2 patients, and five lobes in 2 patients. Abnormalities visible on chest radiography developed in the other 20 patients after admission. The

Table 1. Demographic data and initial clinical signs and symptoms of 76 patients with probable SARS[a]

Data	No. of cases (%)
Sex	
Male	34 (44.7)
Female	42 (55.3)
Age (y) (range, median)	24–87 (46.5)
Underlying disease (no.)	
Nonfatal	62 (81.6)
No underlying disease	52 (68.4)
Mild underlying disease	10 (13.2)
Ultimately fatal	3 (3.9)
Rapidly fatal	11 (14.5)
Initial symptoms	
Fever	76 (100)
Cough	47 (61.8)
Myalgia	37 (48.7)
Dyspnea	31 (40.8)
Diarrhea	24 (31.6)
Rigor	23 (30.3)
Headache	14 (18.4)
Nausea	9 (11.8)
Sore throat	7 (9.2)
Vomiting	3 (3.9)
Rhinorrhea	2 (2.6)

[a]SARS, severe acute respiratory syndrome.

duration from disease onset to the time when abnormalities on the chest radiography were first noted was 1–12 days (median 4 days).

During hospitalization, 69 patients (90.8%) had respiratory distress and needed oxygen supplements. The duration from disease onset to severe respiratory distress was a mean of 9.8 ± 3.0 days (standard deviation [SD]). Endotracheal intubation with ventilator support was indicated for 26 patients, but 3 patients refused intubation. Among the 23 intubated patients, the duration from disease onset to intubation was 8.4 ± 3.3 (SD) days. Eight of these 23 patients were successfully extubated 12.1 ± 6.1 (SD) days later. Twelve of the 23 patients died, and 3 patients remained intubated at the end of the study because of marked lung fibrosis.

Thirty-one patients (40.8%) experienced exacerbation of diarrhea after admission. All had received various antimicrobial agents since hospitalization. The duration from disease onset to severe diarrhea was 8.9 ± 4.7 (SD) days.

During the disease course, leukopenia, lymphopenia, and thrombocytopenia were found in 40, 72, and 61 patients, respectively. Elevation of AST and ALT was noted in 66 and 59 patients, respectively. Elevation of serum LDH, CK, and CRP levels was found in 73, 34, and 71 patients, respectively. The laboratory values of the various parameters listed above typically peaked in severity in the second week of illness (Table 3). The Figure demonstrates the relationships between the time points when several clinical and laboratory parameters became most severe.

New lesions visible on chest radiography developed in 64 patients during hospitalization, including new lesions limited to one lung lobe in 21 patients, two lobes in 13, three lobes in 16, four lobes in 7, and five lobes in 7. The duration from disease onset to the most severe chest radiography findings was 9.6 ± 2.9 (SD) days.

Sputum Gram stain and acid-fast stain, sputum culture for bacteria, sputum chlamydial antigen, throat swab for virus isolation, and urine legionella and pneumococcal antigen tests were available for all 76 patients; all results were negative. Paired serum specimens were available for 41 patients. Tests for antibody reaction to *Mycoplasma*, *Chlamydia*, influenza virus, parainfluenza virus, adenovirus, coxsackievirus, and respiratory syncytial virus were negative. Of these 41 patients, immunoglobulin (Ig) G antibody to SARS-CoV was detected by immunofluorescent assay in 38 patients (92.7%). Twenty-six (34.2%) of the total 76 patients had positive results on throat swab RT-PCR for SARS-CoV. Among the 50 patients whose throat swab results were negative for SARS-CoV by RT-PCR, 28 seroconverted to SARS-CoV. The other 22 patients, who had no direct microbiology or serologic evidence for SARS-CoV infection at the time the study ended, had clinical courses compatible with those of probable SARS and clear relationships as well as exposures to the initial immigrant clusters and the later intrahospital outbreaks in Taiwan (11). Their diagnoses of SARS had also been confirmed by a committee of the Center for Disease Control (Taiwan).

The first patient did not receive any treatment specific for SARS. Five patients did not receive ribavirin treatment because its use was contraindicated for them because of conditions such as cardiac arrhythmia and cardiomyopathy. Seven patients did not receive steroids because their

Table 2. Initial laboratory data of 76 patients with SARS[a]

Laboratory parameters (normal range)	Mean ± standard deviation	No. (%) of patients with abnormal data
Leukocyte counts (4–9 x 10^9/L)	6.0 ± 2.9	
Leukopenia		15 (19.7)
Lymphopenia[b]		49 (64.5)
Platelet counts (150–450 X 0^9/L)	159.7 ± 54.0	
Thrombocytopenia		35 (46.1)
AST (<35 U/L)	36.7 ± 20.0	24[c] (35.3)
ALT (<35 U/L)	27.5 ± 20.4	11[d] (23.9)
LDH (<460 U/L)	597.8 ± 426.0	9[e] (56.3)
CK (<190 U/L)	216.5 ± 444.3	17[f] (26.1)
CRP (<0.8 mg/dL)	3.9 ± 3.6	53[g] (77.9)

[a]SARS, severe acute respiratory syndrome; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; CK, creatine kinase; CRP, C-reactive protein.
[b]Defined as <1 x 10^9/L.
[c]Only 68 patients had been tested.
[d]Only 46 patients had been tested.
[e]Only 16 patients had been tested.
[f]Only 65 patients had been tested.
[g]Only 68 patients had been tested.

Table 3. Data on most severe abnormal laboratory parameters of 76 SARS patients during their disease course[a]

Laboratory parameters (no. of patients, %)	Mean ± SD of most severe data (unit)	Duration from disease onset to most severe data noted
Leukopenia (40, 52.6)	2.5 ± 0.7 (x 10^9/L)	7.5 ± 2.4 days
Lymphopenia (72, 94.7)	0.6 ± 0.3 (x 10^9/L)	7.0 ± 2.5 days
Thrombocytopenia (61, 80.3)	102.3 ± 31.3 (x 10^9/L)	6.9 ± 2.0 days
Elevated AST (66, 86.9)	142.0 ± 323.6 (U/L)	10.3 ± 4.6 days
Elevated ALT (59, 77.6)	103.7 ± 132.2 (U/L)	13.3 ± 5.0 days
Elevated LDH (73, 96.1)	1,323.8 ± 1,487.2 (U/L)	10.8 ± 3.9 days
Elevated CK (34, 44.7)	12,165.7 ± 58,226.9 (U/L)	7.8 ± 4.2 days
Elevated CRP (71, 93.4)	7.1 ± 4.0 (mg/dL)	8.5 ± 3.0 days

[a]SARS, severe acute respiratory syndrome; SD, standard deviation; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; CK, creatine kinase; CRP, C-reactive protein.

cases were rapidly fatal after diagnosis. These seven patients died mainly because of their underlying diseases, especially cardiac events. Their SARS cases did not progress to the point that steroids were indicated according to our treatment protocol.

Eight patients received pulse steroid therapy for progressive clinical conditions, with the usual dosage of steroids. Forty patients received IVIG infusion for severe cytopenia (thrombocytopenia, leukopenia, or both) (22 patients in total) or marked local progression of pulmonary lesions on chest radiography in the first week of disease (18 patients). Those 22 patients who received IVIG for severe cytopenia did not receive steroids while on IVIG; their leukocyte counts were 2.6 ± 1.2 x 10^9/L and 4.3 ± 2.8 x 10^9/L, before and after the use of IVIG, respectively ($p = 0.014$, by paired *t* test). Their platelet counts were 104 ± 35 x 10^9/L and 141 ± 46 x 10^9/L, before and after IVIG, respectively ($p = 0.002$, by paired *t* test).

Various complications developed in 18 patients during hospitalizations, including rhabdomyolysis, peripheral neuropathy, acute renal failure, and fungal or bacterial superinfection (Table 4). Among the 18 nosocomial infections, 6 were bloodstream infections; 3 of these were caused by enterococci, 2 by methicillin-resistant *Staphylococcus epidermidis*, and 1 by methicillin-resistant *S. aureus*. Eleven episodes were lower respiratory infections, diagnosed by the existence of new infiltration on chest radiography, purulent sputum, phagocytosis of bacteria by neutrophils in sputum (indicated by sputum Gram stain), positive sputum culture for bacteria, and response to effective antimicrobial agents. The last episode was a catheter-related infection caused by *Candida parapsilosis*.

The overall death rate was 19.7% (15/76). Among the 14 patients whose underlying diseases were classified as ultimately fatal or rapidly fatal, the rate of death was 78.6% (11/14). For the other 62 patients with mild underlying diseases or without underlying disease, the death rate was 6.5% (4/62). The time from disease onset to death in these 15 patients was 4–42 days (median 12 days). For the 26 patients whose clinical conditions indicated endotracheal intubation with ventilator support, the rate of death was 57.7% (15/26). When we used the logistic regression model for univariate analysis, age, underlying disease (nonfatal versus ultimately or rapidly fatal), initial CRP level, initial absolute neutrophil count (ANC), peak CK level, and peak CRP level were predictors of death. Age, sex, underlying disease, initial chest radiographic findings, initial CRP level, initial ANC, peak CK level, lowest lymphocyte count, worst chest radiographic findings, peak LDH level, and peak CRP level were the predictive factors for respiratory failure (Table 5). However, when we used logistic regression for multivariate analysis, underlying disease and initial CRP level were the only two factors significantly predictive for death (odds ratio [OR], 83.333 and 1.447 every 1 mg/dL increase, respectively; $p < 0.001$ and $p = 0.006$, respectively), and age, initial CRP level, and worst chest radiographic findings were predictive for respiratory failure (OR 1.076, 1.419 every 1 mg/dL, and 2.501 every one-lobe involvement, respectively; $p = 0.01$, $p = 0.01$, and $p = 0.006$, respectively). For the 65 patients who received steroids as the treatment protocol described above, 3 remained febrile and needed further pulse steroid therapy after the use of methylprednisolone with the dosage of 2 mg/kg/day. For the other 62 patients who became afebrile after steroid was administered, 12 had

Table 4. Complications found in 76 SARS patients[a]

Complication	No. of patients (%)
Rhabdomyolysis	4 (5.3)
Peripheral neuropathy	5 (6.6)
Acute renal failure	3 (3.9)
Gastrointestinal bleeding	2 (2.6)
Acute myocardial infarction	1 (1.3)
Bacteria superinfection	10 (13.2)
Candida parapsilosis superinfection	1 (1.3)
MRSA	4 (5.3)
MRSE	2 (2.6)
Enterococci	3 (3.9)
Acinetobacter baumannii	3 (3.9)
Klebsiella pneumoniae	2 (2.6)
Enterobacter cloacae	1 (1.3)
Serratia marcescens	1 (1.3)
Total	18 (23.7)

[a]SARS, severe acute respiratory syndrome; MRSA, methicillin-resistant *Staphylococcus aureus*; MRSE, methicillin-resistant *Staphylococcus epidermidis*. Some patients experience more than one complication.

Table 5. Univariate analysis of factors predictive for death and respiratory failure (logistic regression)

Factors	Odds ratio		p value	
	Mortality	ResF	Mortality	ResF
Age (every 1 y)[b]	1.093	1.082	<0.001	<0.001
Sex (male to female)	3.086	2.841	0.064	0.036
Severe underlying disease[c]	3.973	3.023	<0.001	<0.001
Initial CRP level[b,c] (every 1 mg/dL)	1.300	1.391	0.004	<0.001
Initial ANC count (every 0.1 x 10^9/L)	1.022	1.031	0.020	0.006
Initial lymphocyte count (every 0.1 x 10^9/L)	0.091	0.916	0.188	0.162
Initial platelet count (every 100 x 10^9/L)	0.668	0.565	0.485	0.248
Initial CXR findings (every one-lobe involvement)	1.482	1.941	0.154	0.025
Peak CRP level (every 1 mg/dL)	1.193	1.385	0.033	<0.001
Lowest lymphocyte count (every 0.1 x 10^9/L)	0.801	0.804	0.079	0.032
Peak LDH level (every 100 U/L)	1.132	1.160	0.124	0.008
Peak CK level (every 1 U/L)	1.093	1.082	<0.001	<0.001
Lowest platelet count (every 100 x 10^9/L)	0.724	0.541	0.608	0.261
Worst CXR findings (every one-lobe involvement)	1.505	2.147	0.064	<0.001

[a]ResF, respiratory failure; CRP, C-reactive protein; ANC, absolute neutrophil count; CXR, chest radiography; LDH, lactate dehydrogenase; CK, creatine kinase.
[b]Factors that remained statistically significant after multivariate analysis for respiratory failure.
[c]Factors that remained statistically significant after multivariate analysis for death.

rebound of fever 2–3 days after the temporary defervescence. Seven of the 12 patients became afebrile again and had no fever 1–2 days after the transient rebound of fever without specific intervention. The other five patients received further pulse steroid therapy to control the fever and exacerbated clinical symptoms. For those 62 patients alive at the end of this study, the time to defervescence after disease onset was 10.3 ± 5.1 days. Major lung fibrosis directly caused by SARS developed in 6 of these 62 patients; this condition resulted in exertional dyspnea in 2 patients, oxygen-supplement dependence in 1 patient, and respiratory failure in 3 patients. By the end of the study, 58 of the 62 patients had been successfully discharged from NTUH. The other four patients, including three patients who had respiratory failure and one who was dependent on oxygen supplement, remained hospitalized. The duration of follow-up for these four patients was >4 weeks.

Discussion

Our study of 76 patients with probable SARS with pneumonia demonstrated a high case-fatality rate (19.7%), especially in patients with major underlying diseases and high initial CRP levels. Those patients who needed endotracheal intubation with ventilator support during their hospitalization also had a high rate of death (57.7%). Various complications developed in a high proportion of patients (23.7%) during their disease.

The yield rate of RT-PCR assay for SARS-CoV was lower (34.2%) than in a previous report (5). This finding might be because only throat swabs, not nasopharyngeal aspirations or stools, were obtained for RT-PCR in the present study.

As in previous reports from other areas (4,5), fever was the most frequent initial symptom in our cases. Compared to those previous reports, more patients in our case series initially had diarrhea (31.6% vs. 1%–19.6%). Therefore, according to our observations, diarrhea may be also considered as an early symptom and clue for SARS. In addition, 18 patients had initial symptoms of diarrhea when fever occurred. Gastrointestinal tract should be considered as another important primary infection site of SARS-CoV.

A previous study reported the temporal progression of clinical and radiologic findings in SARS patients and indicated that several parameters would become more severe in the second and third week of disease (5). Our study had similar findings. Although the exacerbation of diarrhea might be due to the use of antimicrobial agents, the diarrhea improved subsequently without their change or discontinuation. Therefore, exacerbation of diarrhea is more likely due to SARS itself. Our study also demonstrates that most patients' abnormal laboratory findings may become more severe in the second week of disease (Table 3 and Figure).

Our treatment protocol was somewhat different from that suggested by So et al. (6). The timing of steroids was modified according to our experiences in treating the second, third, and fourth patients, whose exacerbation of oxygen demand and chest radiography lesions were not prevented by early steroid use. Moreover, steroids are immunosuppressive. A previous study pointed out that the viral load of SARS Co-V in SARS patients arrived at peak levels at approximately day 10 of disease (5). Steroids were used as an adjunctive therapy for infectious diseases to reduce the severity of inflammatory damage that could occur in the later stage of disease (12). Using steroids was also a risk factor for subsequent nosocomial infection (13). For all these reasons, we delayed the use of steroids. Among the 65 patients who received steroids as the treatment protocol, 15 (23.1%) had rebound or persistence of

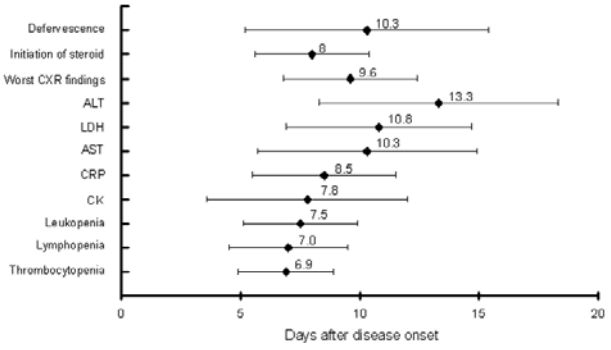

Figure. The time relationships between the time points of defervescence, initiation of steroid, and when chest radiographic finding as well as various laboratory parameters became most severe. Mean and standard deviation (days) are presented. CXR, chest radiography; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; CRP, C-reactive protein; CK, creatine kinase.

fever after initial steroid use. This fever rebound is less frequent than in prior reports (43.3%–85.3%) (5,6). However, the overall death rate in our study was similar to that reported from Hong Kong (7%–20.9%) (3,5). Comparing the treatment results of our study and previous ones is difficult because of different case definitions, patient backgrounds, and disease severity, as well as obscure descriptions about complications in SARS patients in previous reports. All our patients had severe cases. Therefore, the best timing of starting steroid usage and the total duration of steroid usage in SARS patients to improve treatment outcome remain unclear and need further study.

Hemophagocytotic syndrome was documented in our second patient by bone marrow biopsy (14). Her initial clinical signs and symptoms included fever, severe leukopenia, and thrombocytopenia. Her hemophagocytotic syndrome was relieved after using IVIG. This treatment was suggested because of its immune modulating effect (15). The other 21 patients in whom severe leukopenia or thrombocytopenia developed in the first week of disease also received IVIG therapy empirically. IVIG appeared effective for controlling leukopenia and thrombocytopenia: after infusion, the patients' leukocyte and platelet counts increased to a significantly higher level ($p = 0.002$). The increase of leukocyte and platelet counts might have prevented some further complications directly resulting from severe leukopenia and thrombocytopenia, such as infection and tendency to bleed. Although we had no control group, we believe that IVIG may play a role in treating selected SARS patients.

Advanced age, co-existing conditions, high peak LDH level, and high initial ANC count had been reported as factors that predict poor prognosis for SARS patients (4,5,16). By univariate analysis, many parameters predicted death or respiratory failure (Table 3). However, by using the logistic regression model for multivariate analysis, severe underlying disease and high initial CRP level were the only two factors that predicted death; age, initial CRP level, and worst chest radiographic findings predicted respiratory failure. The role of CRP in predicting the outcome of SARS patients has not been discussed in previous studies (4,5,16). The discovery of CRP was reported in 1930 by Tillet and Francis (17). CRP parallels the severity of inflammation or tissue injury and is a useful marker for disease, response to therapy, and ultimate recovery (18,19). Although initial CRP level was not available in eight patients in this cohort, our findings suggest that CRP also parallels well with the severity and outcome of SARS patients. Age and underlying disease were strongly correlated: all our patients with severe underlying disease were older (age >65 years). In a statistical model, these two factors might interfere with each other and lead to the conclusion that age, not underlying disease, was an independent risk for respiratory failure; however, the opposite was true. The worst chest radiographic finding outlined the most severe extent of impaired lung function. This finding might explain why it was an independent factor for respiratory failure. However, other conditions unrelated to pulmonary condition, such as underlying disease or complications during hospitalization, contributed to death. These findings might explain why worst chest radiographic finding was not an independent factor for death.

Forty-two patients in this cohort were admitted to NTUH through the emergency department, which has no facility to check serum LDH level. Also, during intrahospital SARS outbreaks (11), heavy clinical loads and frequent bed transfers made it difficult for the primary care physician to collect laboratory data as the schedule described above. Therefore, initial serum LDH level and CRP level were available for only 16 and 68 patients, respectively; thus, initial LDH level could not be factored into our analyses. Since both CRP and LDH are markers of inflammation, whether the initial LDH level is also an independent risk factor for death or respiratory failure needs further study.

Complications during the disease courses of SARS patients have been seldom or obscurely discussed previously (4,5,16). Acute renal failure, which might be more likely caused by methicillin-resistant S. *aureus* infection and rhabdomyolysis, was found in three patients. Acute myocardial infarction occurred in a patient who had been diagnosed with long-standing coronary artery disease. Gastronintestinal bleeding, which might be due to critical illness, occurred in two patients. Rhabdomyolysis has been reported to be associated with viral infection (20,21). Our observation suggests that SARS Co-V infection might also be associated with this complication (22). Although peripheral neuropathy had also been reported with viral

infection (23), neuropathy caused by steroids or acute illness should also be considered contributing causes in our four patients with neuropathy (24,25).

Eleven patients had bacterial or fungal superinfection during hospitalization. All nosocomial infections occurred while patients were intubated and supported with a mechanical ventilator ($p < 0.001$ by Fisher exact test). The nosocomial infection rate among SARS patients was 237 per 1,000 discharges, which was much higher than that of all patients at NTUH (49 per 1,000 discharges) (26). Steroid use and more severe clinical conditions than usual patients, such as higher rate of respiratory failure, might be the reasons.

Our study shows that SARS has an overall complication rate of 23.7% and case-fatality rate of 19.7%. Clinical symptoms and abnormal radiographic and laboratory findings might become most severe in the second week of disease. In addition to ribavirin and steroids, IVIG may play a role in treating selected patients. Underlying disease and initial CRP level were the two independent predictors of death; age, initial CRP level, and worst chest radiographic finding were the three independent factors predicting respiratory failure for adult SARS patients.

Acknowledgment

We thank Jou-Wei Lin for his contribution on statistics.

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Laboratory Diagnosis of SARS

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The virologic test results of 415 patients with severe acute respiratory syndrome (SARS) were examined. The peak detection rate for SARS-associated coronavirus occurred at week 2 after illness onset for respiratory specimens, at weeks 2 to 3 for stool or rectal swab specimens, and at week 4 for urine specimens. The latest stool sample that was positive by reverse transcription–polymerase chain reaction (RT-PCR) was collected on day 75 while the patient was receiving intensive care. Tracheal aspirate and stool samples had a higher diagnostic yield (RT-PCR average positive rate for first 2 weeks: 66.7% and 56.5%, respectively). Pooled throat and nasal swabs, rectal swab, nasal swab, throat swab, and nasopharyngeal aspirate specimens provided a moderate yield (29.7%–40.0%), whereas throat washing and urine specimens showed a lower yield (17.3% and 4.5%). The collection procedures for stool and pooled nasal and throat swab specimens were the least likely to transmit infection, and the combination gave the highest yield for coronavirus detection by RT-PCR. Positive virologic test results in patient groups were associated with mechanical ventilation or death ($p < 0.001$), suggesting a correlation between viral load and disease severity.

Severe acute respiratory syndrome (SARS) is a new human disease caused by a novel coronavirus, SARS-associated coronavirus (SARS-CoV) (1–5). In Hong Kong, the first recognized outbreak of SARS occurred in early March 2003 in the Prince of Wales Hospital (6,7). Subsequently, outbreaks were reported from other hospitals and from the community (8). As of September 26, 2003, 8,098 cases had been reported to the World Health Organization (WHO) from 29 cities, 1,755 of which were found in Hong Kong (9). No new cases have been found in Hong Kong since June 11, 2003, and on June 23, 2003, WHO removed Hong Kong from the list of areas with local transmission (10). Since identification of the culprit virus in late March 2003, a network has been set up in Hong Kong to provide centralized laboratory diagnostic services for patients with suspected cases of SARS. The diagnostic approach was based on a combination of serologic testing, reverse transcription–polymerase chain reaction (RT-PCR), and virus isolation. Here, we report our experience with the laboratory diagnosis for SARS-CoV infection during this outbreak in Hong Kong, with emphases on the viral shedding pattern, the diagnostic yield of various specimen types, and detection methods.

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Materials and Methods

Patients

This retrospective study analyzed laboratory records of patients admitted to six public hospitals in Hong Kong during the SARS epidemic from March to June 2003. The first inclusion criterion was serologic evidence of SARS-CoV infection. Altogether, 433 patients who exhibited either seroconversion or a fourfold rise in anti–SARS-CoV immunoglobulin (Ig) G antibody titer were identified. Detection of anti-SARS-CoV IgG antibody was based on an in-house immunofluorescence assay that used virus-infected cells. Of the 433 patients with positive serologic test results, 18 were excluded because no samples had been collected for virus detection. As a result, 415 patients were included in this study. Twelve were pediatric patients 3–16 years of age (mean 11.3, standard deviation [SD] 4.1), divided equally between girls and boys. Three hundred thirty-five were adult patients 17–64 years of age (mean 37.1, SD 11.2), with 60.9% females. The remaining 68 were elderly patients 65–97 years of age (mean 76.7, SD 8.2), with 37 (54.4%) women. Altogether, 48/335 (14.3%) of the adult group and 2/68 (2.9%), respectively, of the elderly group required ventilation and received intensive care but recovered; 4/335 (1.2%) of adults and 21/68 (30.9%) of elderly patients died of the infection. All children recovered without requiring mechanical ventilation or intensive care.

Specimen Collection

Respiratory, stool, and rectal swab specimens were collected in viral transport medium, and urine samples were

transported in sterile containers. For some patients, throat and nasal swab samples were pooled into a single specimen container and processed as a single specimen. These samples were referred as "pooled throat and nasal swabs" for the purpose of analysis in this study. Specimens collected were refrigerated (approximately 10°C) until delivery, which were done on the same day in most circumstances. Specimens were kept in iceboxes during delivery to the designated centralized laboratory. SARS-CoV investigations were performed on fresh specimens without prior freezing and thawing.

Viral RNA Detection

SARS-CoV detection by RT-PCR was conducted in two laboratories based on the same primer set COR-1 (sense) 5′ CAC CGT TTC TAC AGG TTA GCT AAC GA 3′, and COR-2 (antisense) 5′ AAA TGT TTA CGC AGG TAA GCG TAA AA 3′ (11). The specimens were centrifuged at 10,000 x *g* for 1 min, and 140 µL of the supernatant were used for RNA extraction using the QIAamp viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Reverse transcription of RNA was conducted in a 20-µL reaction mix containing 4.2 µL of extracted RNA preparation, 2.5 µmol/L of random hexamer and 50 U of reverse transcriptase (Applied Biosystems, Foster City, CA). After incubation at room temperature for 10 min and at 42°C for 30 min, the reaction was stopped by heating at 95°C for 5 min and chilled on ice. The subsequent PCR was conducted in a 50-µL reaction mix containing 5 µL of cDNA template, 1 µmol/L of each primer COR-1 and COR-2, 1.5 U Taq polymerase (Amersham Biosciences, Uppsala, Sweden), 0.2 mmol/L of each deoxynucleoside triphosphate and 2.0 mmol/L magnesium chloride. The cycling conditions were 94°C for 3 min; 45 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR amplicons were visualized by ethidium bromide staining after agarose gel electrophoresis.

The same RNA extraction method was used in laboratory B. The RT-PCR was carried out in a single-tube system (Superscript One-Step RT-PCR with Platinum Taq, Invitrogen, Carlsbad, CA), in a 25-µL reaction mix containing 0.6 µmol/L of each COR-1 and COR-2 primer, 0.2 mmol/L of each deoxynucleoside triphosphate and 1.2 mmol/L magnesium sulphate. The reverse transcription was conducted at 54°C for 30 min. After the mixture was held at 94°C for 3 min, it underwent 45 cycles of amplification at 94°C for 45 sec, 60°C for 45 sec, 72°C for 45 sec, and final extension at 72°C for 7 min. The PCR amplicon was also detected by agarose gel electrophoresis as in laboratory A.

All reagent preparation, sample extraction, amplification, and amplicon detection procedures were conducted in separate areas and under strengthened precautions to avoid cross-contamination. The lower detection limit of the RT-PCR assays was determined by testing preparations with known copies of SARS-CoV as determined by real-time RT-PCR. Both laboratories showed a lower detection limit of 50 viral copies per reaction. In all test runs, positive controls containing approximately 100 copies of viral RNA in viral transport medium were included, and double distilled water was used as a negative control. Positive samples were confirmed by repeating the RNA extraction and RT-PCR from the original samples.

Virus Isolation

Virus isolation for SARS-CoV was performed in laboratory B. Specimens were injected into African green monkey (Vero E6) cell monolayers. For stool or rectal swab samples, the suspension was passed through a 0.45-µm filter before injection. The cell culture tubes were examined daily for diffuse, refractile, rounding cytopathic effects characteristic of SARS-CoV. When cytopathic effects were observed, the cells were stained by the indirect immunofluorescence technique with a convalescent-phase serum sample collected from a SARS patient. The identity of the isolate was further confirmed by RT-PCR.

Statistical Analysis

Statistical tests were performed by using the Statistical Package for the Social Sciences software (SPSS 10.1.0, Inc., Chicago, IL). The chi-square test was used to analyze categorical variables. All statistical tests were two-tailed and p values ≤ 0.05 were regarded as significant.

Results

Specimen Profile

Altogether, 624 respiratory specimens, 671 stool or rectal swab specimens, and 314 urine specimens were collected from the 415 study patients for RT-PCR; 738 respiratory, 810 stool or rectal swab, and 531 urine specimens were submitted for virus isolation; and 558 respiratory, 318 stool or rectal swab, and 296 urine specimens were tested by both RT-PCR and virus isolation. The mean number of specimens collected from each patient was 5.3 (range 1–32, SD 5.1). The mean time of collection of the first specimen was 13.5 days (range 1–88, SD 16.5) after the onset of symptoms (Figure 1). Patients whose first specimens were collected at a later stage of illness had become ill early in the outbreak when no diagnostic test was available.

Shedding Profile

To analyze the profile of viral shedding, specimens were grouped into categories: respiratory, stool or rectal

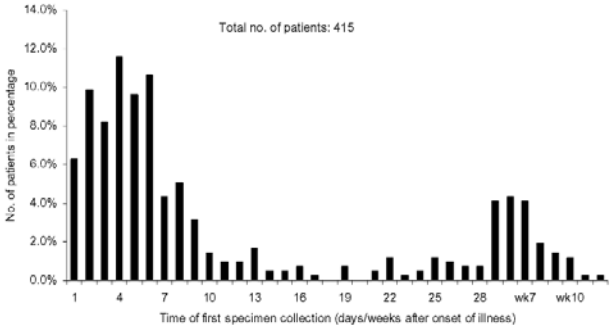

Figure 1. Time of first specimen collection.

swab, and urine. Respiratory specimens included tracheal aspirate, nasopharyngeal aspirate, throat swab, throat washing, nasal swab, and pooled throat and nasal swabs. The viral shedding profile is shown in Figure 2; the number of specimens tested is shown in Table 1. Stool/rectal swab specimens provided the highest positive rate by RT-PCR, followed by respiratory and urine specimens. The RT-PCR positive rate for respiratory specimens increased slightly from week 1 to week 2 after the onset of illness and then dropped to lower levels at week 3 and week 4. The positive rate for stool/rectal swab peaked at week 2 and week 3 and then dropped sharply. The positive rate for urine specimens increased gradually and peaked at week 4. Viral shedding beyond week 6 was rare, with only three stool samples (collected on day 54, day 67, and day 75, respectively) and one respiratory sample collected on day 50 positive by RT-PCR. As for virus isolation, the latest positive specimen was collected on day 31 for a respiratory specimen, day 23 for a urine specimen, and day 6 for a stool sample.

The RT-PCR results of specimens collected within the first 3 weeks after the onset of illness were analyzed to further clarify the viral shedding pattern. The positive rate for respiratory specimens began to increase on day 5 and remained high during the second week. The positive rate for stool/rectal swab specimens peaked at days 9 and 10 and remained high during the second and third week, whereas the detection rate for urine specimens started to increase at the end of the second week (Figure 3).

Diagnostic Yield

The RT-PCR and virus isolation results of different specimen types collected within the first 4 weeks after the onset of symptoms are shown in Table 2. When RT-PCR was used for virus detection, tracheal aspirate and stool provided a high diagnostic yield, with an average positive rate of 66.7% and 56.5%, respectively, for the first 2 weeks. Pooled throat and nasal swabs, rectal swab, nasal swab, throat swab and nasopharyngeal aspirate provided a moderate yield with average positive rates ranging from 29.7% to 40.0% for the first 2 weeks, whereas throat washing and urine specimens provided a lower yield with an average positive rate of 17.3% and 4.5%, respectively. The yield from virus isolation was much lower than from RT-PCR, and no specimen was positive by culture but negative by RT-PCR.

RT-PCR Versus Isolation

To compare the sensitivity of RT-PCR and virus isolation for detecting SARS-CoV, a subgroup analysis was performed on 1,172 specimens that had been submitted for both RT-PCR and virus isolation. The isolation/RT-PCR index, defined as the number of isolation-positive specimens per RT-PCR-positive specimens, was highest for respiratory samples, particularly for pooled throat and nasal swabs, tracheal aspirate, and nasopharyngeal aspirate. The isolation/RT-PCR index for stool or rectal swab samples was approximately 5- to 10-fold lower when compared with that for respiratory specimens (Table 3).

Positive Rate by Patient

Altogether, 132 (31.8%) of the 415 study patients had SARS-CoV detected by RT-PCR or virus isolation. To analyze factors associated with positive virologic testing results, a subgroup analysis was performed on 342 patients whose first specimens were collected within 4 weeks of illness onset. Within this subgroup, 128 (37.4%) patients had one or more positive results by RT-PCR or virus isolation. The mean number of positive specimens among these patients was 1.8 (range 1–10, SD 1.7). The characteristics of patients with and without positive specimens are shown in Table 4. A higher positive detection rate for SARS-CoV was observed for patients with more severe disease ($p < 0.001$ by chi-square test).

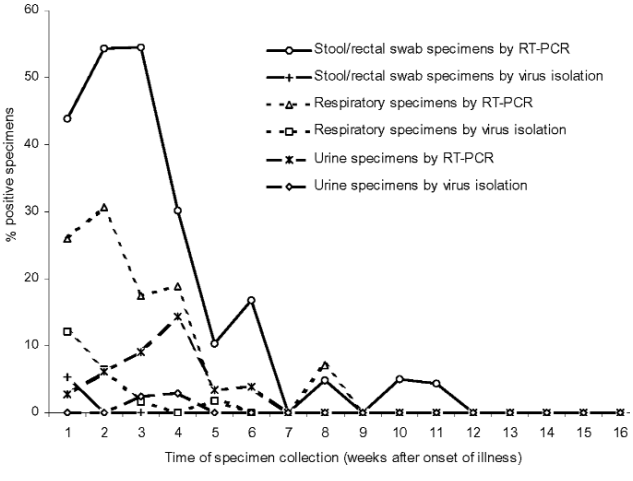

Figure 2. Positive rates of specimen groups according to time of collection from onset of symptoms. The number of specimens tested is shown in Table 1.

Table 1. Specimens tested after onset of illness

	Specimen type					
	Stool/rectal swab		Respiratory		Urine	
Collection time (wk)	RT-PCR[a]	Virus isolation	RT-PCR	Virus isolation	RT-PCR	Virus isolation
1	32	38	243	280	75	110
2	35	40	134	153	82	86
3	44	84	57	62	33	41
4	43	92	37	36	21	35
5	96	113	41	57	29	64
6	110	123	30	50	26	72
7	80	84	18	30	9	38
8	54	55	14	22	10	33
9	49	52	16	27	6	26
10	34	35	16	12	9	10
11	44	44	10	5	9	11
12	21	21	4	2	2	3
13	16	16	2	1	2	1
14	7	7	2	1	1	1
15	5	5	0	0	0	0
16	1	1	0	0	0	0

[a]RT-PCR, reverse transcription–polymerase chain reaction.

Discussion

Identifying the causal agent of the novel emerging infection, SARS, shortly after recognizing its spread in humans, was a remarkable medical accomplishment. This achievement led to the hope for an accurate laboratory diagnosis to guide patient management and to control the spread of infection. During the course of the outbreak, a few centralized laboratories were set up in Hong Kong. All possible resources were deployed to provide a rapid diagnostic service for SARS patients, and a turnaround time of 24 to 48 hours was achieved for RT-PCR. From our experience, more than half of the patients did not have any positive virologic findings. For these patients, the diagnosis could not be confirmed until a convalescent-phase serum specimen was available at a later stage. Thoroughly understanding the viral shedding pattern, the diagnostic yield of various specimen types, and various detection methods is crucial to improve the diagnostic performance.

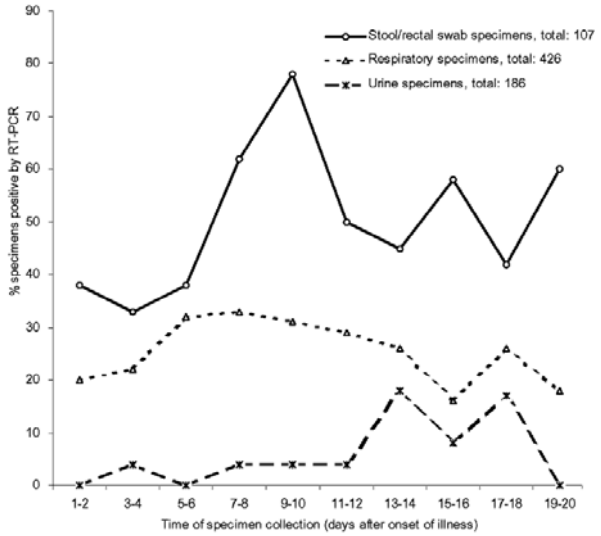

Figure 3. Positive rates of specimens collected within the first 3 weeks.

For most acute respiratory viral infections, the maximal viral shedding occurs in the first few days after illness onset and seldom lasts for more than 10 days (12–14). However, our data indicated that respiratory shedding of SARS-CoV increased over the first week and remained high during the second week. In addition, respiratory shedding >2 weeks after the onset of symptoms was common. This pattern of respiratory shedding is consistent with a previous report of a community outbreak in Hong Kong (15). We found that the peak of viral shedding in stool occurred a few days after that of respiratory shedding. The ability to detect virus in stool specimens peaked at the beginning of the second week and remained high over week 3 and week 4. Occasionally, the shedding of virus in stool could last for more than 6 weeks after the onset of symptoms. The viral shedding peak in urine occurred even later, at weeks 3–4.

In summary, viral shedding of SARS-CoV peaks at a time later than expected and occurs when patients are being hospitalized. This, together with the prolonged viral shedding, could partly explain the propensity for this infection to be transmitted in healthcare settings. We observed that all those who shed virus for a prolonged period (arbitrarily defined as the shedding viruses >6 weeks after onset of symptoms) had their positive samples collected while still critically ill and had received intensive care. The infectiousness of these patients is difficult to discuss because the virus was detected by RT-PCR but not by virus isolation. Nevertheless, further investigations on whether the adverse outcome could be related to inadequate viral clearance are worth pursuing.

Table 2. Diagnostic yield of specimen types to detect SARS-CoV according to time of collection

	No. positive specimens/no. tested for SARS-CoV (%)					
	RT-PCR			Virus isolation		
Specimen type	1 week	2 weeks	3–4 weeks	1 week	2 weeks	3–4 weeks
Respiratory						
Tracheal aspirate	1/2 (50.0)	1/1 (100)	4/4 (100)	2/3 (66.7)	1/1 (100)	0/3
Pooled throat and nasal swabs	6/17 (35.3)	2/3 (66.7)	2/5 (40.0)	4/18 (22.2)	0/3	0/1
Nasal swab	9/27 (33.3)	5/14 (35.7)	1/17 (5.9)	3/29 (10.3)	2/18 (11.1)	0/19
Nasopharyngeal aspirate	39/138 (28.3)	15/44 (34.1)	6/10 (60.0)	23/171 (13.5)	6/54 (11.1)	0/9
Throat swab	5/19 (26.3)	5/14 (35.7)	3/10 (30.0)	2/23 (8.7)	0/15	1/15 (6.7)
Throat washing	4/40 (10.0)	13/58 (22.4)	1/48 (2.1)	0/36	1/62 (1.6)	0/51
Nonrespiratory						
Rectal swab	5/11 (45.5)	2/10 (20.0)	3/7 (42.9)	0/14	0/12	0/35
Stool	9/21 (42.9)	17/25 (68.0)	34/80 (42.5)	2/24 (8.3)	0/28	0/141
Urine	2/75 (2.7)	5/82 (6.1)	6/54 (11.1)	0/110	0/86	2/76 (2.6)

[a]SARS-CoV, severe acute respiratory syndrome–associated coronavirus; RT-PCR, reverse transcription–polymerase chain reaction.

Available data that compare the diagnostic yield of various specimen types are still limited. Wu et al. found that virus was detected in 73% (49/67) of liquid nasopharyngeal gargling samples by a fluorescent PCR (16). However, our data showed that throat washing samples were the most inferior respiratory specimens. In addition to the difference in the sensitivity of detection assays used, the procedures of gargle sample collection could have affected the diagnostic yield. Yam et al. reported that nasopharyngeal aspirate specimens collected between days 1 and 5 after admission provided a similar diagnostic yield when compared to stool samples collected between days 5 and 10 (17). However, data comparing respiratory and stool specimens collected at the same period were not available in their study. In an investigation on a community outbreak in Hong Kong, Peiris et al. reported that respiratory viral shedding peaked during the second week (15). A high positive rate was also obtained from stool samples collected during the second week, but the yield for first week stool samples was not available for comparison.

Nasopharyngeal aspirate is generally regarded as the specimen of choice for detecting respiratory viruses. However, for SARS, the great risk of generating infectious aerosols during the aspiration procedure needs to be considered. We found that pooled throat and nasal swab specimens provided a higher diagnostic yield compared with nasopharyngeal aspirates. Our data indicate that a combination of stool sample and pooled throat and nasal swab specimens should be the specimens of choice for a safe and high-yield SARS-CoV detection. In situations where specimen load is high, pooling of stool sample with throat and nasal swabs for RT-PCR can be considered to minimize the reagent and personnel costs.

SARS-CoV was first isolated from a monkey kidney cell line and is known to produce characteristic cytopathic effects after a few days of incubation in Vero or Vero E6 cell monolayers. At present, the ideal in vitro growth conditions have not yet been elucidated. Our data on isolation/RT-PCR index showed that about 10%–50% of the RT-PCR–positive respiratory and urine specimens had virus grown from Vero E6 cell culture. However, stool and rectal swab specimens had a much lower isolation/RT-PCR index. The presence of toxic substances in stool or rectal swab samples may have interfered with virus isolation. However, toxicity was only occasionally observed on Vero E6 monolayers after adding stool or rectal swab samples.

Table 3. Comparison on positive rates of RT-PCR and virus isolation[a]

	No. (%) of specimens tested positive[b]		
Specimen type (no.)	RT-PCR	Virus isolation	Isolation/RT-PCR index[c]
Pooled throat and nasal swab (30)	8 (26.7)	4 (13.3)	0.50
Tracheal aspirate (13)	6 (46.2)	2 (15.4)	0.33
Nasopharyngeal aspirate (183)	52 (28.4)	14 (7.7)	0.27
Throat swab (58)	11 (19.0)	2 (3.4)	0.18
Nasal swab (56)	14 (25.0)	2 (3.6)	0.14
Urine (296)	14 (4.7)	2 (0.7)	0.14
Throat washing (218)	17 (7.8)	1 (0.5)	0.06
Stool (262)	70 (26.7)	2 (0.8)	0.03
Rectal swab (56)	12 (21.4)	0 (0)	0

[a]RT-PCR, reverse transcription–polymerase chain reaction.
[b]Only specimens tested by both RT-PCR and virus isolation are included.
[c]No. of isolation-positive specimens per RT-PCR-positive specimen.

Table 4. Positive rates for SARS-CoV for various patient groups

Patient characteristics (n)[b]	SARS-CoV result by RT-PCR/virus isolation	
	No. (%) of positive patients[c] (n = 128)	No. (%) of negative patients (n = 214)
Sex		
Female (210)	83 (39.5)	127 (60.5)
Male (132)	45 (34.1)	87 (65.9)
Age group (years)		
≤16 (8)	4 (50.0)	4 (50.0)
17–64 (271)	96 (35.4)	175 (64.6)
≥65 (63)	28 (44.4)	35 (55.6)
No. of specimens tested		
1–2 (116)	39 (33.6)	77 (66.4)
3–5 (111)	36 (32.4)	75 (67.6)
≥6 (115)	53 (46.1)	62 (53.9)
Time of first specimen collected (weeks after illness onset)		
1 (251)	97 (38.6)	154 (61.6)
2 (57)	21 (36.8)	36 (63.2)
3 (11)	4 (36.4)	7 (63.6)
4 (23)	6 (26.1)	17 (73.9)
Disease outcome		
Recovered, not requiring ventilation or intensive care (279)	91 (32.6)	188 (67.4)
Recovered after ventilation or intensive care (40)	22 (55.0)	18 (45.0)
Died (23)	15 (65.2)	8 (34.8)

[a]SARS-CoV, severe acute respiratory syndrome–associated coronavirus.
[b]Only patients with their first specimens collected within 4 weeks of onset of illness are included.
[c]Patients with one or more specimen(s) positive for SARS-CoV by RT-PCR and/or virus isolation.

SARS-CoV can survive for at least 2–4 days at room temperature when mixed with diarrheal or normal stool specimens (18). Thus, the poor isolation rate could not be a result of viral inactivation by fecal contents during specimen storage and transport. The big difference in isolation rate from stool compared to respiratory and urine samples deserve further investigation, and the possibility of viral growth interference due the presence of IgA antibodies needs to be considered.

We found that positive virologic results were associated with more adverse outcomes in patients. This observation could be confounded by the fact that only high-yield specimens, e.g., tracheal aspirate, could be obtained from intubated patients. We verified this point by examining the results of testing other samples from patients with viruses detected from tracheal aspirate samples. We found that all except one of these patients also had viruses detected from other specimen types. Thus, our observations are in line with the fact that more severely affected patients shed a higher load of virus, which facilitated the detection of the virus.

Several options could be considered to improve the ability to accurately diagnose SARS-CoV infection. First, detection of viremia should be included in the diagnostic algorithm because we have found SARS-CoV RNA from blood samples taken within the first few days of onset of symptoms. If this approach is successful, it will close the gap caused by lower virus shedding from the gastrointestinal or respiratory tract that occurs in the first few days after the onset of symptoms. Second, a SARS-CoV-specific monoclonal antibody would be valuable in developing an immunofluorescence assay to detect virus-infected cells from respiratory samples. Such an approach has been shown to provide high sensitivity for influenza and respiratory syncytial viruses. Third, an assay should be developed to detect viral antigens from stool samples as is available for rotavirus detection. Further work to improve the sensitivity and specificity of diagnostic assays for SARS-CoV is needed. The unusual shedding pattern of SARS-CoV should be considered when formulating infection control strategies.

Acknowledgements

We express our appreciation to all healthcare workers in Hong Kong Special Administrative Region who cared for patients with severe acute respiratory syndrome.

Dr. Paul Chan is a clinical virologist and associate professor at the Department of Microbiology, Faculty of Medicine, the Chinese University of Hong Kong. His research interests include emerging viral infection, viral epidemiology, diagnostic virology, and viral oncology.

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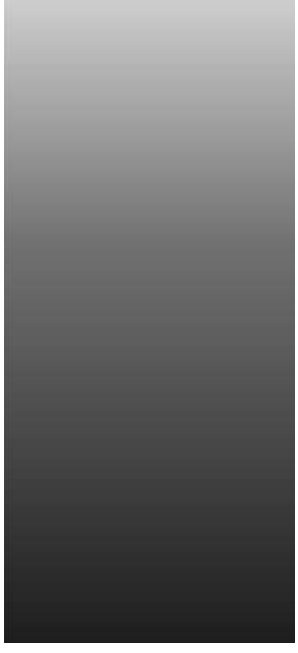

Ring Vaccination and Smallpox Control

Mirjam Kretzschmar,* Susan van den Hof,* Jacco Wallinga,* and Jan van Wijngaarden†

We present a stochastic model for the spread of smallpox after a small number of index cases are introduced into a susceptible population. The model describes a branching process for the spread of the infection and the effects of intervention measures. We discuss scenarios in which ring vaccination of direct contacts of infected persons is sufficient to contain an epidemic. Ring vaccination can be successful if infectious cases are rapidly diagnosed. However, because of the inherent stochastic nature of epidemic outbreaks, both the size and duration of contained outbreaks are highly variable. Intervention requirements depend on the basic reproduction number (R_0), for which different estimates exist. When faced with the decision of whether to rely on ring vaccination, the public health community should be aware that an epidemic might take time to subside even for an eventually successful intervention strategy.

Recently, concerns about a bioterror attack with the smallpox virus or other infectious disease agents have risen (1,2). While new vaccines with fewer adverse consequences are being developed (3), the existing vaccines, which have potential side effects and may be lethal, are the only vaccines available (4,5). In the United States during the recent voluntary smallpox vaccination program, a limited number of healthcare workers volunteered for vaccination because of the risks associated with vaccination and the low for infection (6). If an outbreak occurs, vaccination strategies include ring vaccination around diagnosed cases of smallpox or a mass vaccination to begin as soon as the first cases are diagnosed. Without natural smallpox infections, practical experience with ring vaccination against smallpox cannot be gained; accounts of the vaccination programs that eradicated smallpox in the 1970s are the only source of information (7). Combined with information collected during the last decades of smallpox circulation, mathematical modeling offers a tool to explore various vaccination scenarios if an outbreak occurs (8–15).

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We investigated which conditions are the best for effective use of ring vaccination, a strategy in which direct contacts of diagnosed cases are identified and vaccinated. We also investigated whether monitoring contacts contributes to the success of ring vaccination. We used a stochastic model that distinguished between close and casual contacts to explore the variability in the number of infected persons during an outbreak, and the time until the outbreak is over. We derived expressions for the basic reproduction number (R_0) and the effective reproduction number (R_v). We investigated how effectiveness of ring vaccination depends on the time until diagnosis of a symptomatic case, the time to identify and vaccinate contacts in the close contact and casual contact ring, and the vaccination coverage required to contain an epidemic.

Methods

The model describes the number of infected persons after one or more index cases are introduced. It simulates a stochastic process in which every infected person generates a number of new infections according to a given probability distribution. This process implies that contacts of different infected persons are independent of each other and that no saturation of the incidence occurs at higher prevalence. The model is applicable for the first few generations of infection, if the outbreak goes unchecked, and for the complete outbreak if it is contained. We summarize the main features of the model; the formal model definition is given in the appendix.

Course of Infection and Transmission

The noninfectious state (incubation period plus prodromal phase) lasts 12–15 days (7,16,17) with specified probabilities per day of moving to the infectious state. The assumption that infectivity during the prodromal phase is negligible is supported by a recently published statistical analysis of outbreak data (16). The duration of the infectious state D_I is 14 days (7,16,18), with variable infectiousness during that time (13,19,8). The probability of transmission per contact p_τ, where τ denotes the day of the infectious period, is high at the beginning and low at the

end of the infectious period (Figure 1A). At the end of the infectious period, a person either recovers or dies. The case-fatality rate is 30% (14), which is an average value for the case-fatality rate of variola major.

Transmission takes place in two rings of contacts: 1) household and other close contacts, and 2) more casual face-to-face contacts. We assumed that in the close contact ring the probability of transmission is five times higher than in the casual contact ring (g = 0.2). The number of contacts on day τ of the infectious period in the close contact ring follows a Poisson distribution with mean $\mu_\tau^{(1)}$, and in the casual contact ring this number follows a a negative binomial distribution with mean $\mu_\tau^{(2)}$. The values ($\mu_\tau^{(1)} = 2$ and $\mu_\tau^{(2)} = 14.9$) were chosen such that the total number of contacts per day was comparable to numbers observed in empirical studies (20) (Figure 1B). For every contact, the event of transmission is determined by the infectiousness by day of the infectious period.

Most people can be infected again, and smallpox can develop 10–20 years after vaccination (21,22). While residual immunity might lower the case-fatality rate, it might also lead to a later diagnosis for infected persons because disease symptoms are milder. An infection with milder symptoms is probably less infectious, but an infectious person might have more contacts with others because he or she feels less impaired by disease symptoms. Hence, the net effects of residual immunity are difficult to assess. We assumed that all persons are equally susceptible, and that no protective immunity remains in the population from previous vaccination.

The basic reproduction number R_0 describes the average number of secondary cases produced from contact with an infected person during the infectious period and without intervention. The number can be computed as the sum of the reproduction numbers in the close contact and casual contact ring

$$R_0 = R_0^{(1)} + R_0^{(2)} = \sum_{\tau=1}^{D_I} \mu_\tau^{(1)} p_\tau + g \sum_{\tau=1}^{D_I} \mu_\tau^{(2)} p_\tau$$

For the baseline parameter values given in the Table, R_0 = 5.23, which, when broken down by rings of contacts, gives $R_0^{(1)}$=2.1 and $R_0^{(2)}$=3.3, i.e., 40.2% of all transmissions take place in the close contact ring. We use the parameter a_1 in the function describing the transmission probability (Table) to vary the basic reproduction number, i.e., if we want to simulate an outbreak under the assumption that R_0 is 5, we chose a_1 accordingly. In the literature, the estimates given for R_0 vary between 3–6 (9,10,23) and 10–20 (1,14).

Ring Vaccination

Ring vaccination in the model includes complete isolation of symptomatic patients with diagnosed cases of smallpox and vaccination of (some or) all contacts of the

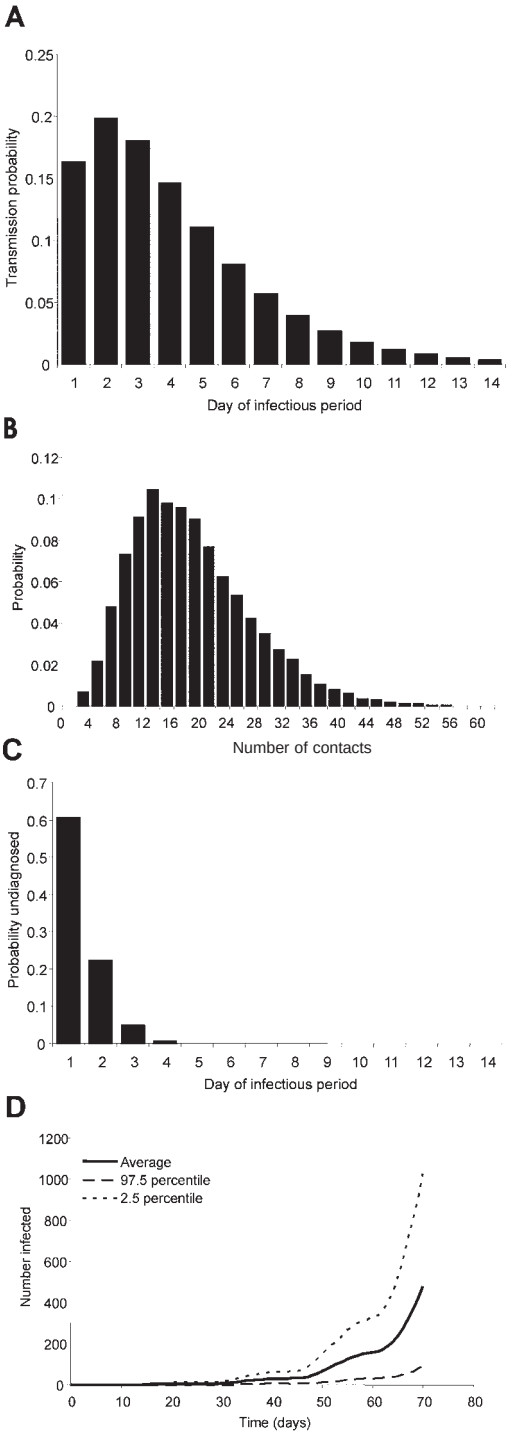

Figure 1. A, the transmission probability per contact by day of the infectious period; B, the probability distribution of the number of contacts with susceptible persons per day; C, the probability of remaining an undiagnosed, but infectious, case by day of the infectious period; and D, the mean (solid line) and the 2.5% and 97.5% percentiles (dotted lines) of the number of infected persons for 500 simulation runs for an epidemic without any intervention after the introduction of one index case at the beginning of this incubation period at t = 0.

Table. The baseline parameter values

Model parameter	Notation	Baseline value
Course of the infection		
Maximum duration latent period	D_E	15 d
Probability of transition to infectious state on day τ of the latent period	γ_τ, $\tau=1,...,D_E$	0.0 for τ = 1,...,12 0.3 for τ = 13 0.6 for τ = 14 1.0 for τ = 15
Duration infectious period	D_I	14 days
Case-fatality rate	f	0.3
Transmission probability per contact	p_τ	$a_1 \tau e^{(-a_2 \tau)}$ with $a_1 = 0.27$, $a_2 = 0.5$
Ratio of infectiousness of casual contact and close contact ring	g	0.2
Contacts		
Mean number of contacts in close contact ring (Poisson distribution)	$\mu_\tau^{(1)}$	2
Mean number of contacts in the casual contact ring (negative binomial distribution)	$\mu_\tau^{(2)}$	14.9 (sd 8.4) NegBin(4, 0.212)
Intervention		
Probability of diagnosis	δ_τ	$1-e^{-b_1(\tau-b_2)}$ with $b_i = 0.5$, $b_2 = 0$ ($b_2 = 6$ for first index case)
Time needed to trace contact	$r^{(i)}$	1 day for i = 1, 3 days for i = 2 (3 days for both rings for first index case)
Time window during which vaccination is effective	w	4 days
Vaccination coverage	$c^{(i)}$	0.95 for i = 1, 0.5 for i = 2 (0.5 for both rings for first index case)

diagnosed case-patient. In our baseline scenario, we assumed that vaccinated contacts are not isolated after vaccination and may therefore transmit the infection to others if they become infectious. In addition, we enhance the baseline intervention by including monitoring of identified contacts. The effectiveness of the intervention therefore is determined by the probability of diagnosis per day of the infectious period, the time needed to identify contacts of the close contact and casual contact ring, the vaccination coverage in the close contact and the casual contact ring, and whether contacts are monitored. Some of those parameters (speed of diagnosis and time to identifying contacts) differ between the first index case in the population and cases occurring later in the epidemic. In Figure 2, the timing of the key events in the chain of transmission and intervention is shown schematically. The index patient can cause new cases of infection between the beginning of the infectious period until diagnosis and isolation. For a secondary case, vaccination has to take place within 4 days after infection (14) to prevent disease.

We denote with δ_τ the probability of diagnosis on day τ of the infectious period for those persons who have not been diagnosed before. From those probabilities, one can derive the probability that an infectious person is not yet diagnosed on day τ of his or her infectious period (Figure 1C). By $\upsilon_\tau^{(i)}$, we denote the probability that a contact in ring i (i = 1 or 2), who was infected on day τ of the index patient's infectious period, will be vaccinated within 4 days of being infected. In the appendix, υ_τ depends on the diagnosis probabilities, the time needed for contact tracing, and the vaccination coverage. Throughout, we assume that the vaccine efficacy is 100%. We can now determine an effective reproduction number R_υ that describes the number of secondary cases caused by an index patient in a situation with intervention:

$$R_\upsilon = \sum_{\tau=1}^{D_I} (\mu_\tau^{(1)}(1-\upsilon_\tau^{(1)}) + g\mu_\tau^{(2)}(1-\upsilon_\tau^{(2)}))\, p_\tau \prod_{j=1}^{\tau-1}(1-\delta_j)$$

A special strategy included in this formula is an intervention where only case isolation is performed without vaccination of contacts. The vaccination coverage $c^{(i)}$ is set to zero. Equivalently, it describes the situation that no window period exists (24). The reproduction number can then be calculated as

$$R_\upsilon = \sum_{\tau=1}^{D_I} (\mu_\tau^{(1)} + g\mu_\tau^{(2)})\, p_\tau \prod_{j=1}^{\tau-1}(1-\delta_j)$$

If vaccination is ineffective, but contacts are monitored, the monitoring will have the same effect on R_0 as an effective vaccination, because the contacts will not be able to disseminate the virus any further (assuming a fully effective monitoring). Therefore, R_υ can be computed with the formula including vaccination, where the window period is now set to w = 15, of the full duration of the infectious period. This assumption means that regardless of when the index patient's condition is diagnosed, contacts can effectively be excluded from further transmission. If monitoring of contacts is not 100% effective, the parameter $c^{(i)}$ for the coverage can be used to express the extent of successful monitoring.

The outbreak can be controlled if $R_\upsilon < 1$. In the Table, the model parameters and their baseline values are listed. In the Appendix, the formal model definition is given.

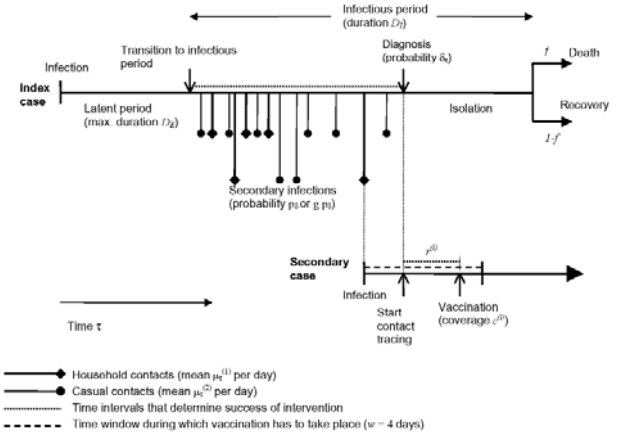

Figure 2.The time course of events in the process of transmission and intervention. The success of intervention is essentially determined by the time between start of the infectious period and diagnosis of the index case, and the time between the start of contact tracing and the vaccination of the contact.

Results

Baseline Parameter Set

An epidemic starting with one index case in a completely susceptible population without intervention grows exponentially, if it survives early extinction. The large range of possible courses of the epidemic reflects the stochastic variability (Figure 1D). If the intervention does not succeed in reducing the effective reproduction number R_υ to below 1, the epidemic will continue to grow exponentially, albeit at a lower rate. For example, if diagnosed infectious persons are isolated, but no ring vaccination is performed, the effective reproduction number is R_υ=1.65 and the epidemic cannot be contained.

If the intervention succeeds in reducing the effective reproduction number R_υ to <1, the size of successive generations of infected persons declines. For the parameter values of the baseline scenario given in the Table, we have R_υ = 0.67 and 94.4% of all transmissions take place in the casual contact ring. The epidemic can then be contained and the virus eradicated. In Figure 3A and B, the distribution of the total number of infected persons (excluding those who were vaccinated in time to prevent symptomatic infection) and of the time until recovery of the last infected patient is shown for 500 simulation runs with the baseline parameters (Table). The time until the epidemic is over is quite variable: on average it takes 82 days, in some cases it takes up to 1 year (range 22–334 days). During this time, an average of 209 contacts are vaccinated (range 6–1,038 contacts), with a mean number of 13.5 vaccinated contacts per infected case. On average, 15 persons are infected (range 1–123 persons), if we exclude those infected persons who were vaccinated on time. If we

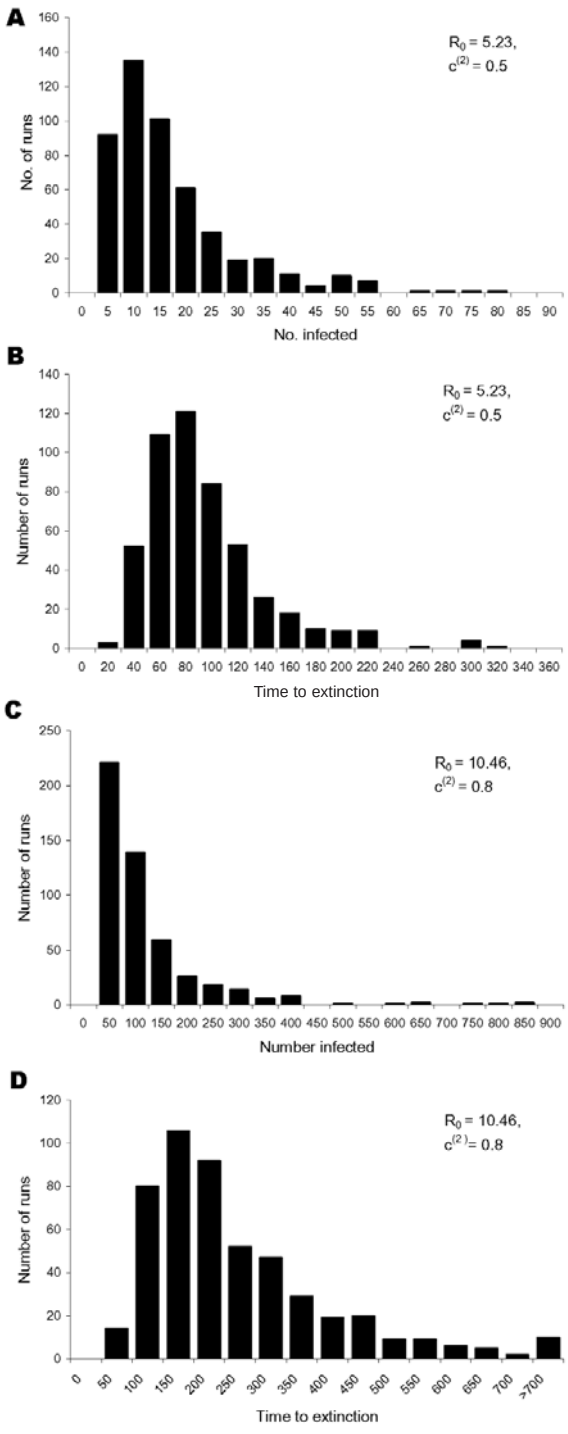

Figure 3. The distribution of A, the total number of infected persons excluding those infected contacts who were vaccinated on time to prevent disease, and B, the time to extinction for 500 simulation runs with the baseline intervention parameter values and a basic reproduction number of 5.23. For a basic reproduction number of 10.46 and an increase of the vaccination coverage in the casual contact ring to 80% in C, the distribution of the total number of infected persons, and in D, the distribution of the time to extinction, is shown for 500 simulation runs.

include the infected contacts who were vaccinated on time, the mean number of persons infected is 29 (range 1–200 contacts). On average, 5 persons die of smallpox (range 0–30 persons). If monitoring of contacts is added to the intervention (incorporated in the model by assuming that $w = 15$), the effective reproduction number can be further reduced to 0.53. The average number of infected persons drops to 6 (range 1–43 persons), excluding the identified infected contacts who are vaccinated or monitored and to 14 (range 1–96 persons), including the vaccinated and monitored infected contacts. The mean time to extinction is now 53 days (range 20–288 days). The fraction of transmissions taking place in the casual contact ring is slightly lower. at 93.7%.

To contrast the baseline scenario, in Figure 3C and D we show results for the case that $R_0 = 10.46$, i.e., twice the value of baseline scenario. To contain the epidemic, we now assumed that 80% of all contacts in the casual contact ring were vaccinated in time. The effective reproduction number R_v was 0.91. A fraction of 91.8% of transmissions took place in the casual contact ring. The mean number of infected persons during the epidemic was 101 (range 2–663), excluding the infected contacts vaccinated in time and 340 (range 2–2,175 persons) including those contacts. The mean time to extinction was 229 days (range 32 to >900 days). The time to extinction can be very long when R_v is near 1 because the epidemic can flare up again when a case by chance produces many secondary infections. A similar picture would result if, in addition to vaccinating casual contacts with a coverage of 55%, those contacts are monitored. The effective reproduction number is then 0.96; 93.0% of transmissions are in the casual contact ring.

Sensitivity Analysis

Initial Phase of Epidemic

The initial phase of the epidemic (time before discovery of the first case) is determined by the number of index patients that start the epidemic outbreak and by the time it takes to diagnose the first case. We varied those two variables separately while assuming that after diagnosis intervention took place within the parameters defined in the Table, i.e., with an R_v of 0.67 (Figure 4). While the number of cases increases almost linearly with the number of index cases, the dependency on the time to diagnosis shows the influence of the variable infectiousness during the infectious period. In the beginning, when infectiousness is high, the number of infected persons increases rapidly. Another rise occurs toward the end of the index patient's infectious period because the second-generation patients become infectious and produce the third generation of infected persons. Once the second generation of

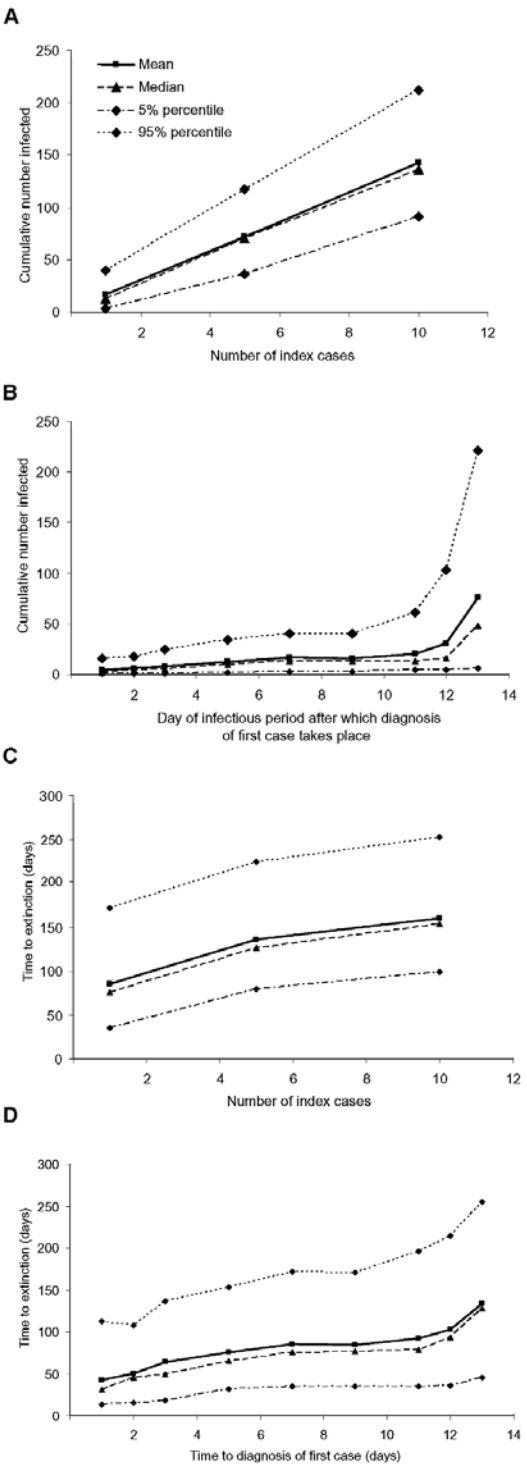

Figure 4. Results for the sensitivity analyses. The total number of infected persons (excluding successfully vaccinated infected contacts) depends on A, the number of index cases starting the epidemic, and B, the day of the infectious period after which the diagnosis of the first case occurs. The time to extinction is shown for C, different numbers of index cases, and D, the day of the infectious period after which the diagnosis of the first case occurs. The quarntiles are taken pointwise for 500 simulation runs.

infected persons has the opportunity to disseminate the infection further, the range of possible outcomes increases greatly (range 6–221 infected persons). A similar picture emerges for the time needed to extinguish the outbreak. The duration of the outbreak increases when diagnosis is delayed during the first few days of infectiousness, then stays on a stable level, and finally increases again when diagnosis is delayed towards the end of the infectious period (Figure 4D). Therefore, diagnosing the first index case before the second generation of infected persons start transmitting the virus is important. If diagnosing the first case at the beginning of its infectious period is possible, the number of cases during the epidemic can be kept at a low level.

Effectiveness of Response

Among others, the value of R_0 determines whether ring vaccination as defined above can contain an epidemic or not. As the value of R_0 is uncertain (2,9,19), we studied R_υ as a function of R_0 (Figure 5). In Figure 5A, an intervention without monitoring of contacts is considered with various assumptions on how long tracing and vaccinating casual contacts take. In this case, ring vaccination can contain the epidemic if R_0 is <7, and contacts can be traced within 3 days. In Figure 5B, monitoring of contacts is added to the intervention. In that instance, the epidemic can be contained up to an R_0 of 10. In Figure 5A and B, we assumed that 50% of all casual contacts can be identified and vaccinated or monitored.

In Figure 6, we show how the critical vaccination coverage needed to control the epidemic depends on R_0, or, more specifically, on the average number of daily contacts (Figure 6C). In addition, we varied the baseline assumption about the time to diagnosis by shifting the probability of being diagnosed by n days towards a later time in the symptomatic period. In Figure 6A, without monitoring of vaccinated contacts, a shift by 1 day greatly increases the coverage needed to contain the epidemic. If diagnosis is delayed by >1 day, the chances of controlling the epidemic diminish greatly. If vaccinated contacts are monitored, the situation improves (Figure 6B), and a high vaccination coverage in the casual contact ring ensures that the epidemic stays under control.

Time to Extinction Depending on R_υ

Finally, we looked at how differences in intervention effectiveness influence the duration of the epidemic and the cumulative number of infected persons. The effective reproduction number R_υ was varied by decreasing the vaccination coverage in the casual contact ring stepwise to 0.1. The effective reproduction number increased up to 0.96 (starting from the baseline value of 0.67). Figures 7A and b show how the cumulative number of infected persons and the time to extinction increase with increasing R_υ. The mean time until extinction approximately doubles to almost 200 days, and the range of possible outcomes increases with maximum possible durations of >2 years. The mean number of infected persons increases by a factor of 5, and the range of possible outcomes increases such that epidemics with several hundreds of infected persons are possible. Hence, if R_υ is slightly <1, the epidemic might take a long time to control, and the number of persons who become infected and die might be high.

Discussion

Our simulation results show that a smallpox epidemic starting from a small number of index cases can be contained by ring vaccination provided the intervention measures are very effective. The time to diagnosis has proven to be an essential and sensitive parameter in determining the intervention effectiveness. The speed of diagnosis is less essential if identified contacts are isolated to prevent them from transmitting further if their vaccination fails. The time window limiting the success of vaccination then loses its importance for determining the effectiveness of intervention. The time to diagnosis of cases and the fraction of contacts found by contact tracing are then the key parameters. Quick contact tracing would be even more essential if substantial transmission would take place during the prodromal period of infection as is assumed by some authors (11,12).

Some limitations of our modeling approach should be kept in mind. First, we only consider epidemics that are started by a small number of index cases. The branching process approach does not allow for overlapping rings of contact, but we implicitly include such an effect by varying the effective transmission probability such that the distribution of transmissions over the infectious period agrees with empiric findings (8). In other words, the decreasing probability of contacting new susceptible persons during the infectious period is incorporated in the decreasing transmission probability per contact. For larger numbers of index cases, our approach can be viewed as a worst-case scenario. Second, we assume that the population is completely susceptible, i.e., no residual immunity from vaccination in the pre-eradication era exists. This lack of immunity means that if previously vaccinated persons cannot become infectious to others, our results are too pessimistic, whereas if they become infectious with mitigated symptoms, our results might be too optimistic.

In the recent literature, other models, both stochastic and deterministic, of smallpox outbreaks have been introduced to analyze the effects of ring and mass vaccination (8,10–14). On the basis of a low estimate for R_0, Meltzer et al. (8) concluded that even quarantine alone can control the epidemic, as can vaccination alone, if the transmission rate is reduced sufficiently. However, the model does not allow

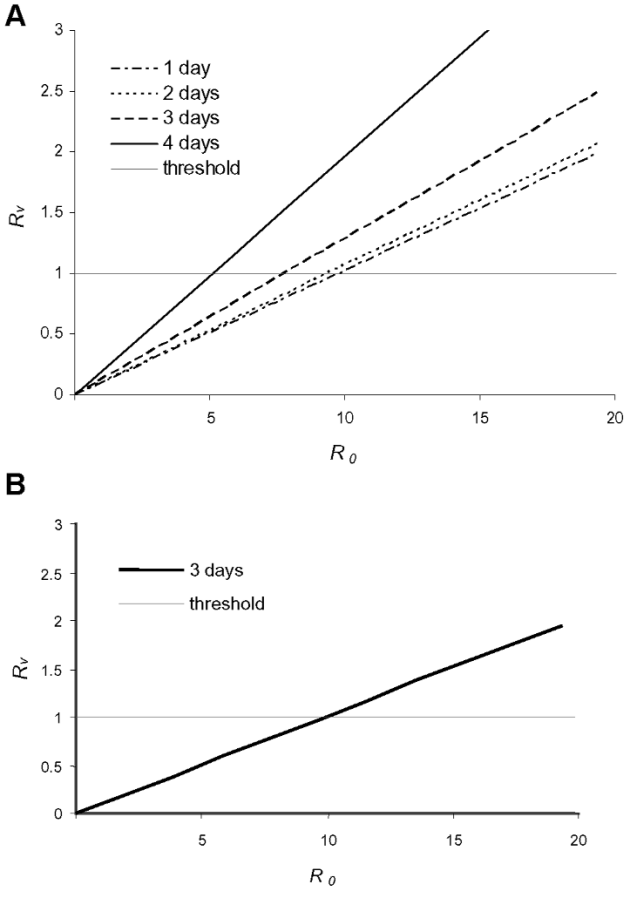

Figure 5. The effective reproduction number R_v, that determines the success of intervention is shown as a function of the basic reproduction number R_0 for a vaccination coverage of 50% in the casual contact ring. In A, contacts are not monitored after vaccination; in B, all identified contacts are isolated and cause not further transmission. The different lines in A are for different assumptions about how long it takes to trace and vaccinate those contacts. In B, it does not make a difference whether it takes 1, 2, or 3 days to find the contacts. If R_0 is 5, the intervention will be successful in both cases, if R_0 is 10, 50% coverage is no longer sufficient to curb the epidemic.

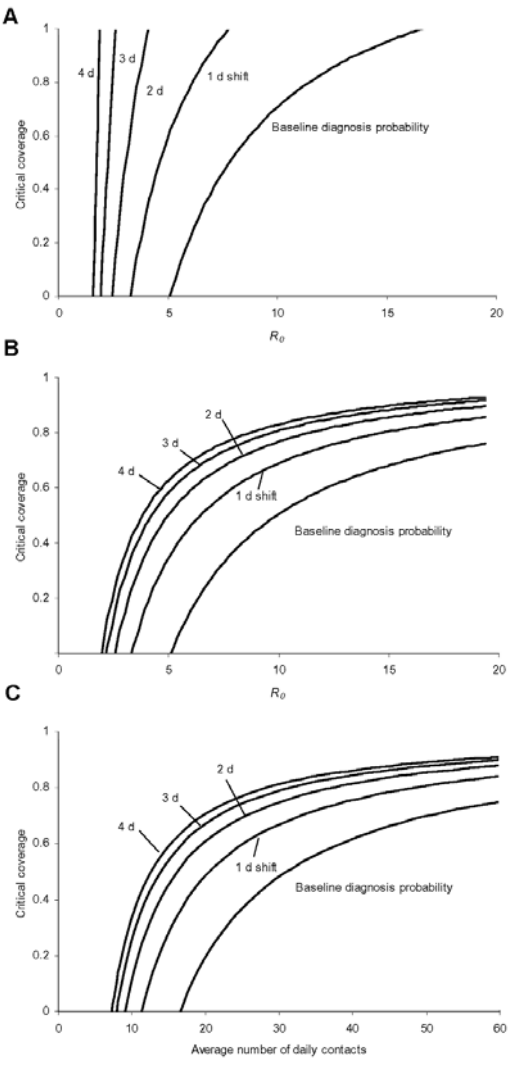

Figure 6. Here the critical vaccination coverage in the casual contact ring is shown as a function of the basic reproduction number R_0 for different assumptions about the time it takes to diagnose infectious persons. A, for the baseline assumption, that diagnosis is very quick after the beginning of the infectious period, a low coverage is sufficient if R_0 is 5, but for R_0 around 10 the coverage has to be at least 70% for the intervention to be successful. If the probability of being diagnosed shifts to later days of the infectious period, the situation quickly gets out of control and vaccination can no longer curb the epidemic. In B, the same is shown with the difference that here we assume that vaccinated contacts are successfully monitored such that they can no longer produce any secondary infections, even if their vaccination was too late to prevent them from becoming infectious. In this case a later diagnosis is not that influential, but nevertheless, if R_0 is 10, the vaccination coverage (or the percentage of contacts identified and monitored) must be at least 50% to guarantee success. In C, the critical coverage of the casual contact ring is shown as a function of the average number of contacts per day, again for the situation where vaccination is combined with monitoring of contacts. The average number of contacts was varied by varying the number of daily casual contacts. The effect is similar to that of varying R_0 through the transmission probability per contact as shown in B.

analysis of how intervention parameters determine the reduction of the transmission rate. On the other hand, Kaplan et al. (10) conclude that with a large number of initial cases, mass vaccination will prevent more deaths than will a vaccination strategy based on contact tracing. Those authors explicitly take into account the limited resources available for tracing and vaccinating contacts, a limitation that is an important factor in large outbreaks. Also, they assume that infectivity is high during the prodromal phase. As in the model by Kaplan et al. (10), our model takes into account that the intervals between infection and diagnosis of an index case, and between diagnosis of an index case and tracing of the contact, may exceed the time window in

which vaccination has to take place. This window limits the possible effectiveness of contact vaccination—a phenomenon termed "race to trace" by Kaplan et al. While the model by Kaplan et al. is based on differential equations with exponentially distributed sojourn times in different compartments, our model is a stochastic model that is able to deal with more realistic distributions for sojourn times in different disease states. Also, our model can provide estimates for variability in outcomes (Discussion in [25]).

In a study by Halloran et al. (11), a stochastic model for smallpox outbreaks in small, structured communities is described. In some respects the model is similar to ours, namely, that there is a distinction between household contacts and other contacts in the community with differing transmission probabilities. The values of most biologic parameters are choices similar to ours, with the exception of Halloran's assumption that persons are highly infectious during the prodromal phase. An important difference between the models is the natural limitation of the number of infected persons in any epidemic attributable to the rather small community size in Halloran's model. The nonlinear effects of saturation play a rather large role in determining the outbreak size, especially for less effective intervention measures. Also, Halloran's model seems to be too complex to derive an explicit formula for the basic reproduction number R_0, and thus makes sensitivity analysis of the results based on that quantity much more tedious. One of the main differences, however, is the way that vaccination is incorporated into the model does not allow investigation of the effects of those parameters that largely determine the success of intervention, namely the time to diagnosis of new cases and the time needed to trace contacts. In a small closed community as the one described in the model, tracing of contacts is not that difficult, but in modern society with its increasing mobility the tracing of casual contacts can pose a big problem.

The main difference between the modeling approach of Bozette et al. (12,13) and our model is that in Bozette's R_0 and R_υ are set to prescribed values, while in our model those numbers can be derived from measurable quantities inherent to the transmission and intervention process. In comparison to the estimate of R_υ of 0.53 in our baseline scenario with vaccination and monitoring of contacts, Bozette et al. assume a much lower value of 0.1. So compared to our results, their results are rather optimistic, but they cannot relate the assumed value of R_υ to R_0 or to parameters describing the process of contact tracing and vaccination.

Finally, Eichner (14) recently published a modeling study that uses a simulation model to assess the effectiveness of case isolation and contact tracing. Modeling approach and choice of parameter values resemble our approach, but the intervention is modeled in a more phenomenologic way by defining the outcomes of intervention without explicitly including intervention-related parameters into the model. This does not allow for an explicit calculation of the effective reproduction number based on intervention parameters as is possible in our approach.

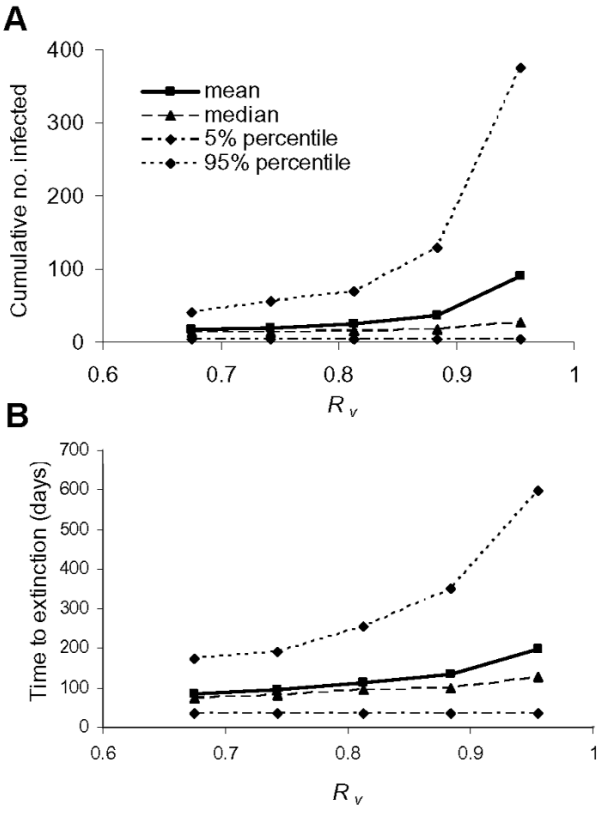

Figure 7. A, the cumulative number of infected persons (excluding successfully vaccinated infected contacts), and B, the time to extinction are shown for various values of the effective reproduction number R_υ. The quantiles are taken pointwise for 500 simulation runs.

With respect to preparing for a smallpox outbreak, alertness and ability to diagnose quickly are important. Physicians and nurses need to be educated and the public needs to be more aware. Also, since we know little about the timing and effectiveness of identifying infectious persons and their contacts in case of a bioterror attack, obtaining more empirical information about contact patterns and contact tracing will be helpful. Recently, some useful data about contact patterns have been collected during severe acute respiratory syndrome outbreaks, but a more systematic investigation of contact tracing is advisable. Considering the uncertainties connected to all parameter values, we conclude that any contingency plan for use of ring vaccination must also identify the criteria under which switching to large-scale mass vaccination is justified.

Acknowledgments

We thank three anonymous referees for their helpful and constructive comments.

Dr. Kretzschmar is a senior research scientist at the Department of Infectious Diseases Epidemiology of the National Institute of Public Health and the Environment in Bilthoven, the Netherlands. She is developing mathematical models for the spread and prevention of infectious diseases. She has worked on sexual network structure and the spread and prevention of sexually transmitted diseases and HIV/AIDS and on vaccine-preventable diseases.

Appendix

We used a stochastic model to describe the number of infected persons following the introduction of one or more index cases. The model simulates a discrete time-branching process.

Formal Model Definition

By $E_{t,\tau}$ we denote the number of persons infected at time t-τ who are still latent at time t, $1 \le \tau \le D_E$. By $I_{t,\tau}$ we denote the number of persons who became symptomatic at time t-τ and are not yet in isolation at time t, $1 \le \tau \le D_I$. By $Q_{t,\tau}$ we denote the number of persons who became symptomatic at time t-τ and are in isolation at time t, $1 \le \tau \le D_I$.

S_τ denotes a Bernoulli distributed random variable with mean γ_τ, $1 \le \tau \le D_E$, where γ_τ is the probability to move to the infectious state on day τ of the latent period. D_E is the maximum duration of the latent period.

T_τ denotes a Bernoulli distributed random variable with mean p_τ, $1 \le \tau \le D_I$, where p_τ is the transmission probability per contact on day τ of the infectious period. D_I is the duration of the infectious period. We assume that p_τ can be well described by the functional form $p_\tau = a_1 \tau e^{(-a_2 \tau)}$ with $a_1, a_2 > 0$.

$C_\tau^{(1)}$ denotes a Poisson distributed random variable with mean $\mu_\tau^{(1)}$, $1 \le \tau \le D_I$ describing the number of contacts per day in the close contact ring.

$C_\tau^{(2)}$ denotes a random variable with a negative binomial distribution with parameters n_τ and q, $1 \le \tau \le D_I$ describing the number of contacts per day in the casual contact ring. The mean number of contacts per day in the casual contact ring is $\mu_\tau^{(2)} = n_\tau(1-q_\tau)/q_\tau$ and the standard deviation is $\sigma_\tau = \sqrt{n_\tau(1-q_\tau)}/q_\tau$.

The transition from being undiagnosed infectious to isolation depends on the probability of diagnosis Δ_τ on day τ after the start of the infectious period. Δ_τ is a Bernoulli distributed random variable with parameter δ_τ, $\tau = 1,\ldots,D_I$.

$V_\tau^{(1)}$ and $V_\tau^{(2)}$ are Bernoulli distributed random variables with parameters $\upsilon_\tau^{(1)}$ and $\upsilon_\tau^{(1)}$, respectively, with $\tau = 1,\ldots,D_I$. They describe the probability that a contact in ring 1 or 2 , respectively, will be effectively vaccinated within the time window of 4 days after infection. The subscript τ refers to day of the infectious period of the index case at the moment of transmission. The vaccination probabilities $\upsilon_\tau^{(i)}$ for i=1,2 depend on the probability that the index case is diagnosed and on the vaccination coverage as follows. The probability $\overline{\delta_{\tau,j}}$ an infectious person who has transmitted to a contact on day τ of the infectious period is diagnosed on day τ+j can be computed as

$$\overline{\delta_{\tau,j}} = \delta_{\tau+j} \prod_{k=\tau}^{\tau+j-1} (1-\delta_k) \text{ for } j=1,\ldots, D_I-\tau+1.$$

The probability for the infected contact to be vaccinated in time depends on the number of days *w* after infection that vaccination can still be effective, the number of days $r^{(i)}$ that are needed for tracing the contact, and the coverage $c^{(i)}$ (the fraction of contacts in ring i that are effectively immunized) for i=1,2. Then we get

$$\upsilon_\tau^{(i)} = \sum_{k=1}^{Min(D_I-\tau+1,\tau+w-1-r^{(i)})} \overline{\delta_{\tau,k}} \; c^{(i)} \text{ for i=1,2.}$$

To describe the probability of diagnosis per day of the infectious period we use the functional form

$$\delta_\tau = 1 - e^{-b_1(\tau-b_2)} \text{ for } \tau > b_2, \text{ and } \delta_\tau = 0 \text{ for } \tau < b_2$$

with $b_1 > 0$, and an integer b_2 between 0 and D_I. In the simulations different values for those parameters are chosen for the first index patient, who starts the epidemic, and later cases assuming that it takes longer to diagnose the first case as there is not yet that much alertness of the public health system. Also, the time needed to find contacts and the vaccination coverage may vary between the first index case and later cases.

The transitions through the different stages in time is described by the system of difference equations.

$$E_{t+1,\tau+1} = E_{t,\tau} - \sum_{k=1}^{E_{t,\tau}} S_{k,\tau}, \quad \tau = 1,\ldots, D_E$$

$$I_{t+1,\tau+1} = I_{t,\tau} - \sum_{k=1}^{I_{t,\tau}} \Delta_{k,\tau}, \quad \tau = 1,\ldots, D_I$$

$$Q_{t+1,\tau+1} = Q_{t,\tau} + \sum_{k=1}^{I_{t,\tau}} \Delta_{k,\tau}, \quad \tau = 1,\ldots, D_I$$

The inflow of new latent and symptomatically infected persons is given by

$$E_{t+1,1} = \sum_{\tau=1}^{D_I} \sum_{k=1}^{I_{t,\tau}} \left(\sum_{n=1}^{C_\tau^{(1)}} T_{k,n,\tau}(1-V_{k,n,\tau}^{(1)}) + \sum_{n=1}^{C_\tau^{(2)}} T_{k,n,\tau}(1-V_{k,n,\tau}^{(2)}) \right)$$

$$I_{t+1,1} = \sum_{\tau=1}^{D_E} \sum_{k=1}^{E_{t,\tau}} S_{k,\tau}$$

The initial conditions for the case that the epidemic is started by one infected index case entering the population at the beginning of his latent period are given by

$E_{0,1} = 1$,
$E_{0,\tau} = 0$ for $\tau = 2,\ldots, D_E$,
$I_{0,\tau} = 0$ for $\tau = 1,\ldots, D_I$, and
$Q_{0,\tau} = 0$ for $\tau = 1,\ldots, D_I$.

At the end of the infectious period an infected patient either recovers and becomes immune or dies. Death occurs in a fraction *f* of all cases, i.e., *f* denotes the case fatality. This implies that the number of deaths M_{t+1} at time *t+1* is given by

$$M_{t+1} = \sum_{k=1}^{I_{t,D_I}} Z_k \ ,$$

where Z is a Bernoulli distributed random variable with parameter *f* , the case-fatality rate. The cumulative mortality from the start of the epidemic up to time t is given by $\overline{M}_t = \sum_{n=1}^{t} M_n$.

The model was implemented and run in Mathematica 4.2.

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Virulence Factors for Hemolytic Uremic Syndrome, Denmark[1]

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We present an analysis of strain and patient factors associated with the development of bloody diarrhea and hemolytic uremic syndrome (HUS) among Shiga toxin–producing *Escherichia coli* (STEC) patients registered in Denmark in a 6-year period. Of 343 STEC patients, bloody diarrhea developed in 36.4% and HUS in 6.1%. In a multivariate logistic regression model, risk factors for bloody diarrhea were the *eae* and stx_2 genes, O groups O157 and O103, and increasing age. Risk factors for HUS were presence of the stx_2 (odds ratio [OR] 18.9) and *eae* (OR undefined) genes, being a child, and having bloody diarrhea. O group O157, although associated with HUS in a univariate analysis (OR 4.0), was not associated in the multivariate analysis (OR 1.1). This finding indicates that, rather than the O group, the combined presence of the *eae* and *stx*2 genes is an important predictor of HUS.

Shiga toxin–producing *Escherichia coli* (STEC), alternatively known as verocytotoxin–producing *E. coli*, is a major cause of gastroenteritis in developed countries (1). Symptoms range from mild to severe gastroenteritis with bloody diarrhea, and the infection may be complicated with hemolytic uremic syndrome (HUS) (1). STEC strains are defined by the Shiga (Stx) toxins; two classes, Stx1 and Stx2, are recognized (2). STEC strains also frequently harbor the locus of enterocyte effacement (LEE) pathogenicity island, which encodes intimin and a number of other virulence factors responsible for the intestinal attaching and effacing lesions (2). Several other factors may be involved in the pathogenic process, among them the enterohemolysin produced by many STEC strains (2). In several regions of the world, STEC strains of O group O157 are isolated from most patients, but STEC strains may belong to a large number of other O groups (2,3).

Only a few epidemiologic studies have addressed the relative importance of virulence factors for serious clinical disease. We studied a cohort of Danish STEC patients and determined risk factors for HUS and bloody diarrhea among a series of microbiologic and patient-related characteristics.

Materials and Methods

Patients and Isolates

From January 1, 1997, to May 1, 2003, 425 patients with STEC infections were registered in Denmark, a country of 5.4 million. These 425 registrations comprised the instances in which an STEC strain had been isolated from a fecal specimen obtained from a patient. In Denmark, clinical specimens for analysis for bacterial gastrointestinal pathogens are submitted to the Statens Serum Institut (SSI) or to 1 of 10 regional clinical microbiologic laboratories, depending on the county in which the physician who requests the analysis resides. In most patients (92%), infections were diagnosed from specimens sent to SSI, where the screening procedure was based on presence of the *stx* genes. Briefly, the diagnostic procedure consisted of isolating bacterial strains by using the SSI enteric media (4) and screening colonies with an *E. coli* morphology for *stx* genes by using colony hybridization with pooled DNA polynucleotide probes.

Subsequently, all isolates were referred to the national reference laboratory at SSI, and the STEC diagnosis was verified by using the Vero cell assay (5). The isolates were then further characterized for reference purposes. This process included full O:H serotyping (6) and determining the presence of the stx_1, stx_2, *eae* (intimin encoding), and *ehxA* (enterohemolysin encoding) virulence genes by hybridization to individual DNA polynucleotide probes.

Patient Interviews

Starting January 1, 1997, STEC patients registered in Denmark have been routinely interviewed by physicians at the Department of Gastrointestinal Infections at SSI. This practice was initiated to identify possible outbreaks and to

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[1]Presented in part at the VTEC2003, 5th International Symposium on "Shiga Toxin (Verocytotoxin) Producing *Escherichia coli* Infections," Edinburgh, Scotland, June 2003 (abstract O-6).

obtain data on the basic manifestations of disease and routes of infection. The patients (or their parents) were contacted by phone and interviewed by using a concise questionnaire. The questions concerned information on signs and symptoms, treatment, travel, contact with animals and other persons with similar symptoms, and whether the person, before becoming ill, had consumed items from a short list of foodstuffs and drinks that might potentially be associated with STEC infections. These interviews were the prime source of deciding whether the patients had HUS or bloody diarrhea, although for some patients, information from the requesting physicians was also obtained.

Patient Data

A total of 82 patients were excluded from the cohort for various reasons. The isolates from 11 patients were not fully characterized with regard to virulence factors. For another 62 patients, details from interviews were insufficient, usually because patients could not be contacted by phone or because of language difficulties. Finally, two different STEC strains of different O groups were found in the fecal samples of nine patients. These strains were excluded because determining which isolate was responsible for the clinical symptoms was not possible. Among these nine patients were two with HUS. These were girls, ages l and 2 years, one of whom had bloody diarrhea. The girls were infected with isolates of O groups O145 and O156, and O groups O21 and O145, respectively. All four strains were *eae*-positive, stx_1-negative, and stx_2-positive.

Thus, the dataset used in the present analyses contained 343 patients. Nearly all of the illnesses were sporadic since no general outbreaks occurred in Denmark within the study period. A few patients may have been epidemiologically linked, however, by belonging to the same family or attending the same school or kindergarten.

The dataset was analyzed with respect to the two types of clinical outcomes, HUS and bloody diarrhea, by using a set of possible and available explanatory variables consisting of both microbiologic and patient factors. The microbiologic factors were O group and the absence or presence of each of the *eae*, stx_1, stx_2, and *ehxA* genes. By definition all isolates contained one or both of the stx_1 and stx_2 genes. The patient factors were age and gender; whether the patient had been hospitalized; diarrhea; bloody diarrhea (when analyzing for HUS); fever; vomiting; abdominal pain or joint pains; whether the patients had received antimobility agents or antimicrobial agents as a result of the infection; acquisition of the infection abroad; patient contact with other persons with similar symptoms or with animals (and if so which animals); and whether or not the patient, in the 5 days before symptoms developed, had eaten at a restaurant or consumed beef, lamb, pork, poultry, bean sprouts, unpasteurized milk, apple juice, or unwashed raw vegetables.

Statistical Analyses

The statistical analyses were performed by using the SAS statistical package (Cary, NC). Variables were considered in a logistic regression analysis on the basis of statistical significance in a univariate analysis and biological relevance. Variables were subsequently eliminated from the logistic regression models if they were not significant at the 5% level or not confounding other explanatory variables; competing models were compared with likelihood ratio testing. Finally, the models were examined for statistical interactions, and odds ratios (ORs) with 95% confidence intervals (CIs) were calculated.

All variables were dichotomous, except age. While the age distribution for the whole group of STEC patients and for patients with bloody diarrhea was only slightly skewed towards younger age groups, the HUS patients were young; of the 21 HUS patients, 18 were <8 years of age. For this reason, age was entered as an interval scale variable in the model for bloody diarrhea but as a dichotomous variable in the model for HUS, comparing the patients aged ≥8 years to those ≤7 years.

Results

The cohort consisted of 343 patients; HUS had developed in 21 (6.1%), and bloody diarrhea had occurred in 125 (36.4%). The isolates comprised 74 serotypes and 49 different O groups. Among the O groups, O157 was the most common, comprising 81 (23.6%) isolates followed by O26 with 50 (14.6%) isolates and O103 with 45 (13.1%) isolates. A total of 30 different O groups occurred only once. Table 1 summarizes the O groups and the occurrence of HUS, bloody diarrhea, virulence genes, and median age of patients within the O groups. Of the patients, 32.2% had been hospitalized, 16.2% had received antibimicrobial treatment, and 16.4% had received treatment with antimobility agents as a result of the STEC infection; 22.0% reported having been infected on foreign travel.

HUS

Among the 21 strains isolated from HUS patients, 11 were O group O157; 3 were O26; 2 were O111; and 1 each was O103, O121, O137, O145, and O165. Of the O157 strains, nine were O157:H7 and two O157:H-. All of the 21 strains were positive for the *eae* gene, while 18 strains were stx_2-positive, 1 strain stx_1-positive, and 2 strains stx_1- and stx_2-positive. Eighteen of the patients were ≤7 years of age. Table 2 shows the occurrence of bloody diarrhea, virulence genes, and median age of the patients within the O groups of the isolates from the 21 HUS patients.

Table 1. Number of isolates, clinical outcome, virulence factors, and median age for the O groups of STEC patients, Denmark[a]

O group	No. of isolates	HUS	Bloody diarrhea	*eae*-positive	stx_1-positive	stx_2-positive	*ehxA*-positive	Median age
O157	81	11	56	80	31	81	80	12
O26	50	3	16	50	42	12	45	1
O103	45	1	21	44	45	0	44	4
Other[b]	30	2	8	12	17	17	12	8
O rough	21	0	4	4	16	15	14	28
O117	19	0	1	0	19	0	1	32
O145	18	1	5	17	7	11	18	3
O146	18	0	4	0	13	15	11	48
O121	9	1	3	9	0	9	9	1
O91	7	0	0	0	6	5	4	25
O nontypeable	7	0	0	1	4	3	3	25
O111	6	2	2	6	6	3	5	1
O128	6	0	0	0	6	5	5	31
O156	5	0	0	2	5	0	2	32
O55	4	0	1	2	4	0	0	3
O15	3	0	1	0	0	3	1	21
O70	3	0	0	3	2	1	3	1
O174	3	0	2	0	2	2	2	36
O5	2	0	1	1	2	0	2	30
O41	2	0	0	1	2	0	0	40
O76	2	0	0	0	2	0	1	42
O88	2	0	0	0	1	2	2	39
All	343	21	125	232	232	184	264	12

[a]STEC, Shiga toxin–producing *Escherichia coli*; HUS, hemolytic uremic syndrome.
[b]Includes all O groups that contained one isolate only.

ORs for each of the available possible determinants were calculated. On the basis of the possible determinants that were significantly associated with HUS within 90% confidence limits, a multivariate logistic regression model for HUS development was built. Because all strains from HUS case-patients contained the *eae* gene, the OR of this determinant could not be estimated, and the final model contained just three determinants: presence of the stx_2 gene, bloody diarrhea, and age of ≤7 years. No statistical interaction was detected in the model. The details of the results of the univariate and multivariate analyses of the odds of developing HUS are shown in Table 3. Notably, O group O157, though significant in the univariate analysis, was not a risk factor. The OR for O157 declined from 4.0 (95% CI 1.6 to 9.7) to 1.1 (95% CI 0.4 to 3.1) when the other factors were included in the model. Of the other predictors, O group O111 was also associated with an increased risk for HUS in the univariate analysis but not in the multivariate analysis (Table 3).

Eight of the HUS patients and 43 of the non-HUS patients had received antimicrobial treatment during the course of the STEC infection, and in fact the variable of being treated with antimicrobial agents as a result of the infection was also associated with an increased risk for HUS in a univariate analysis (OR 3.6; 95% CI 1.4 to 9.1) and when included in the multivariate analysis (OR 4.8; 95% CI 1.4 to 16.2). However, the antimicrobial treatment status was unknown for 29 of the non-HUS patients, so they were excluded from the analysis when the variable was included. The variable did not confound or interact with the three other determinants when added to the model.

The two remaining microbiologic traits, presence of the stx_1 gene and the *ehxA* gene, were not associated with an increased risk for HUS in the univariate or multivariate analyses. Apart from the fact that all HUS patients had been hospitalized, the remaining available patient characteristics (i.e., sex, foreign travel, other symptoms apart from bloody diarrhea, contact with animals or other ill persons, and the food and drink exposures) were also not associated with increased risk for HUS.

Bloody Diarrhea

An analysis was also made of risk factors for bloody diarrhea. On the basis of the patient interviews and the available clinical information, 125 patients with bloody diarrhea were identified. Of the strains isolated from these patients, 86% contained the *eae* gene and 67% the stx_2 gene; 45% of the strains belonged to O group O157 and 17% to O group O103.

Again, ORs for each of the available putative determinants were calculated, and a logistic regression model was developed on the basis thereof. The final model contained the following five variables: *eae*-positive, stx_2-positive, O group O157, O group O103, and age. Age was incorporated as an interval scale variable. The modeling indicated

Table 2. Number of isolates, instances of bloody diarrhea, virulence factors, and median age for O groups among 21 patients with hemolytic uremic syndrome

O group	No. of isolates	Bloody diarrhea	*eae*- positive	stx_1-positive	stx_2-positive	*ehxA*-positive	Median age
O157	11	8	11	0	11	10	4
O26	3	2	3	0	3	2	1
O111	2	2	2	2	2	1	1
O103	1	1	1	1	0	1	1
O121	1	0	1	0	1	1	1
O137	1	0	1	0	1	1	3
O145	1	1	1	0	1	1	2
O165	1	1	1	0	1	0	0
All	21	15	21	3	20	17	1

that all the above-mentioned variables were associated with an increased risk for bloody diarrhea (Table 4). Presence of the *eae* gene was the virulence factor that showed the closest association with development of bloody diarrhea, whereas the effect of presence of the stx_2 gene was more modest. In contrast to what was found regarding HUS, O group O157 and O103 were independent risk factors for bloody diarrhea. In addition, there was statistical interaction ($p = 0.03$) between the determinants *eae*-positive and O group O103. However, only one O103 strain was *eae*-negative, and on this basis we choose to ignore this interaction.

Age was found to be a risk factor for bloody diarrhea, but the risk increased with age as opposed to what was found for HUS patients (Table 4). In addition, there was an association (multivariate OR 2.2; 95% CI 1.1 to 5.0) between bloody diarrhea and indigenous infection (i.e., not infected while traveling abroad), although the status of this variable was unknown in 14 instances (data not shown). Finally, 29 of 117 bloody diarrhea patients and 22 of 197 nonbloody diarrhea patients had received antimicrobial treatment as a result of the STEC infection and, as for the analysis for HUS, the OR of antimicrobial treatment could be estimated after reducing the dataset by 29 cases. The univariate OR was 2.6 (95% CI 1.4 to 4.8), and upon inclusion of this variable in the logistic regression model, the multivariate OR was 3.0 (95% CI 1.4 to 6.3).

Virulence factors not associated with increased odds of bloody diarrhea included presence of the stx_1 gene (not shown) and presence of the *ehxA* gene, although the latter, on the basis of the univariate analysis alone, was included as a risk factor (Table 4). The OR for presence of the *ehxA* gene was 0.9 (95% CI 0.4 to 1.9) when this variable was included in the model. None of the remaining patient characteristics was associated with bloody diarrhea in the multivariate analysis, although two symptoms were associated in the univariate analysis, namely fever (OR 1.8; 95% CI 1.1 to 2.9) and abdominal pains (OR 2.6; 95% CI 1.4 to 4.9). In addition, bloody diarrhea conferred an increased risk for hospitalization (OR 6.7; 95% CI 4.0 to 11.3).

Table 3. Risk factors for HUS among 343 STEC patients, Denmark, 1997–2003[a]

			Univariate analysis		Multivariate analysis	
Determinant	No. of patients	No. (%) with HUS	OR	95% CI	OR	95% CI
eae						
Negative	111	0	1			
Positive	232	21 (9.1)	ND[b]		NI	
stx_2						
Negative	159	1 (0.6)	1		1	
Positive	184	20 (10.9)	19.3	2.6 to 145	18.9	2.4 to 146
Age						
≥8 y	178	3 (1.7)	1		1	
≤7 y	165	18 (10.9)	7.5	2.2 to 26.0	11.4	3.2 to 41.3
Bloody diarrhea						
No	218	6 (2.8)	1		1	
Yes	125	15 (12.0)	4.8	1.8 to 12.8	4.5	1.6 to 12.7
O157						
No	262	10 (3.8)	1			
Yes	81	11 (13.6)	4.0	1.6 to 9.7	NS	
O111						
No	337	19 (5.6)	1			
Yes	6	2 (33.3)	8.4	1.4 to 48.6	NS	

[a]STEC, Shiga toxin–producing *Escherichia coli;* HUS, hemolytic uremic syndrome; OR, odds ratio; CI, confidence intervals; NI, not included (test not appropriate); ND, not defined; NS, not statistically significant.
[b]$p < 0.0002$, Fisher exact test.

Table 4. Risk factors for bloody diarrhea among 343 STEC patients, Denmark, 1997–2003

			Univariate analysis		Multivariate analysis	
Determinant	No. of patients	No. (%) with BD	OR	95% CI	OR	95% CI
eae						
Negative	111	17 (15.3)	1		1	
Positive	232	108 (46.6)	4.8	2.7 to 8.6	6.0	2.7 to 13.3
stx_2						
Negative	159	41 (25.8)	1		1	
Positive	184	84 (45.7)	2.4	1.5 to 3.8	2.5	1.2 to 5.1
O157						
No	262	69 (26.3)	1		1	
Yes	81	56 (69.1)	6.3	3.6 to 10.8	2.7	1.2 to 5.7
O103						
No	298	104 (34.9)	1		1	
Yes	45	21 (46.7)	1.6	0.9 to 3.1	2.8	1.2 to 6.3
ehxA						
No	79	15 (19.0)	1			
Yes	264	110 (41.7)	3.0	1.7 to 5.6	NS	
Age[b]	–	–	–	–	1.3	1.2 to 1.5

[a]STEC, Shiga toxin–producing *Escherichia coli;* BD, bloody diarrhea; OR, odds ratio; CI, confidence intervals; NS, not statistically significant.
[b]Calculated for an increase in age of 10 years.

Discussion

This study presents an analysis of determinants of serious clinical disease among Danish STEC patients during a period of 6.33 years. The dataset we used stems from a full national cohort of largely sporadic cases. The strains have been collected and the patients interviewed prospectively. Furthermore, for >90% of the strains, the diagnostic screening methods have been based on presence of the *stx* genes, the very feature that distinguishes STEC from other *E. coli*. The dataset is therefore largely free of several types of otherwise common biases, such as those that may potentially arise through the process of selecting strains from strain collections, from studying outbreak strains, through missing patient information, or through surveillance directed towards certain serotypes such as O157:H7. For these reasons we find this dataset, although relatively small, to be well suited for an analysis of the impact of basic patient characteristics and virulence factors—in particular, the significance of different serotypes or O groups—on serious disease.

However, the limitations of the dataset should also be kept in mind. Among these is the relatively small number of available explanatory variables. At present, we have data on only a small series of virulence genes and no information on subtypes of individual genes. Also, the basic nature of the available patient information does not permit any type of detailed analysis of many patient-related factors that may play a role in the development of serious symptoms.

More than three quarters of the patients and roughly half of those complicated with HUS were infected with non-O157 STEC strains. The determinants associated with the development of HUS were the stx_2 gene, the *eae* gene (indicating the presence of the LEE pathogenicity island), and young age. In addition, bloody diarrhea and treatment with antimicrobial agents were independently associated with HUS. O group O157 was not associated with an increased risk for HUS when we controlled for the presence of the stx_2 gene. The apparent association with O157 seen in the univariate analysis was primarily due to the fact that all the O157 isolates contained the stx_2 gene. Thus, our findings indicate that the potential of *E. coli* O157 strains to cause HUS may by and large be explained by the fact that these strains almost invariably encode Stx2 and the virulence factors of the LEE pathogenicity island.

The association between HUS and the *eae* gene, the stx_2 gene, or both, has been observed in several other epidemiologic studies (7–10), although to our knowledge only one has been based on multivariate modeling (11). Although the approach taken in that study was somewhat different from ours, the conclusions were in line with ours; the authors found that, of a series of virulence genes including the *ehxA* gene, the *eae* and stx_2 genes were the only significant determinants of HUS. Two other recent studies also point to the association between the *eae* and stx_2 genes and the development of HUS. One compares virulence genes in non-O157 STEC strains from HUS patients with those from other infected persons in the United Kingdom (12); the other compares the frequency with which the two genes and the various O groups occur among German STEC patients with and without HUS (13).

In the analysis for bloody diarrhea, the determinants we found were, again, presence of the *eae* and stx_2 genes, although the stx_2 association was much less pronounced. In addition, the two most frequently occurring O groups, O157 and O103, were independent determinants. The latter finding indicates that additional virulence factors

important for inducing bloody diarrhea may be present among isolates of these two O groups. Since bloody diarrhea was a risk factor in the analysis for HUS, this may also be of some importance for the development of HUS. We note that the single stx_2-negative STEC strain isolated from an HUS patient was of O group O103.

In concordance with the findings of others (2), our results do not indicate that the *ehxA* gene plays an independent role in the pathogenic process leading to HUS or bloody diarrhea. The *ehxA* gene was not associated with HUS and although, on the basis of the univariate analysis, it appeared to be associated with bloody diarrhea, this association was no longer present in the multivariate analysis, i.e., after controlling for the determinants present in the model for bloody diarrhea.

Treatment with antimicrobial agents was a risk factor for both HUS and bloody diarrhea. This finding is of interest in light of the ongoing discussion of whether or not certain antimicrobial agents have the potential to induce serious clinical symptoms including HUS among patients with STEC infections (14). However, our data do not have sufficient clinical detail to determine the causal direction of this finding. Patients with HUS or bloody diarrhea initially have more severe symptoms than other STEC patients and may be more likely to receive antimicrobial treatment. Therefore, we may be observing a selection for more severe symptoms and not an effect of the antimicrobial agents per se.

Finally, we note that although only 9 patients out of the full cohort of 425 patients had two different STEC isolates simultaneously recovered from their stools, these 9 patients comprised 2 of the 23 HUS patients known to be present within the cohort. In both instances, both isolates were *eae*- and stx_2-positive. This finding raises the possibility that different STEC isolates may complement each other in the pathogenic process leading to HUS and that such synergy may lead to an increased risk of developing HUS.

To conclude, our analyses indicate that the determinants associated with development of HUS are presence of the *eae* gene, presence of the stx_2 gene, being a young child, and bloody diarrhea. Our analyses also indicate that the combined presence of the *eae* and stx_2 genes is a better marker for the potential to cause HUS than is O group O157. This finding may have implications for the future planning of improved diagnostics and surveillance of STEC infections.

Acknowledgments

We thank Kristiane Beicher for restructuring and maintaining the department *Escherichia coli* databases.

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Enzyme-linked Immunosorbent Assay and Serologic Responses to *Pneumocystis jiroveci*

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Seroepidemiologic studies of *Pneumocystis* pneumonia (PCP) in humans have been limited by inadequate reagents. We have developed an enzyme-linked immunosorbent assay (ELISA) using three overlapping recombinant fragments of the human *Pneumocystis* major surface glycoprotein (MsgA, MsgB, and MsgC) for analysis of antibody responses in HIV-positive patients and healthy blood donors. HIV-positive patients had significantly higher antibody levels to all Msg fragments. Furthermore, HIV-positive patients who experienced a previous episode of PCP (PCP-positive) had higher levels of antibodies to MsgC than patients who never had PCP. A significant association was found between ELISA antibody level and reactivity by Western blot in HIV-positive patients, especially those who were PCP-positive. Thus, this ELISA will be useful in studying serum antibody responses to *Pneumocystis* in different human populations.

Pneumocystis is an opportunistic fungus of worldwide distribution that can cause lethal pneumonia in persons infected with HIV and in other persons with depressed immune function (1). *Pneumocystis* also infects a variety of animals and causes pneumonia in those that are immunodeficient or immunosuppressed (2). Since *Pneumocystis* organisms in humans and animals are very similar, and the pneumonia that develops has many common features, these microbes were originally thought to belong to a single genus and species. However, data over the past decade have shown that *Pneumocystis* is genetically diverse and host-specific, suggesting that studies of immune responses to *Pneumocystis* are best performed by using organisms or organism products that are specific for that host (1). *Pneumocystis* nomenclature, which is evolving and somewhat controversial, has designated two species so far: *P. carinii* for rat-derived organisms and *P. jiroveci* for human-derived organisms (3).

Seroepidemiologic surveys have shown that exposure to *Pneumocystis* occurs early in life; by 2 to 4 years of age, most children have antibodies to the organism (4–8). Since a reliable in vitro culture system for *Pneumocystis* has not been developed, antigens used in these studies consisted mainly of whole or fractionated organism preparations derived from infected human or rodent lungs. The use of human *Pneumocystis* has been further hindered by the small amounts of material that can be obtained from clinical specimens. Serologic surveys using crude *Pneumocystis* antigen preparations are generally not effective as clinical or epidemiologic tools. The frequency or level of serum antibodies to *Pneumocystis* among HIV-positive patients and other immunocompromised hosts has usually been similar to the corresponding value in healthy controls (6,8–15). Conflicting results have been obtained in attempts to distinguish past from present infection or colonization from active disease (16–21). Thus, no standardized antigen preparations are available for antigen-specific immunologic studies of *Pneumocystis* infection in humans.

The *Pneumocystis* antigen that has received the most attention is the 95- to 140-kDa major surface glycoprotein (Msg or gpA), which contains shared and species-specific epitopes, elicits humoral and cellular protective immune responses, and plays a central role in the interaction of *Pneumocystis* with its host (2–8,22). However, native *P. jiroveci* Msg is in short supply and contains multiple isoforms of this glycoprotein, which complicates immunologic studies of this antigen (23,24).

Recombinant *Pneumocystis* antigens offer a viable approach to developing novel reagents for use in immunologic assays (25–27). One group of investigators has developed two recombinant fragments that correspond to

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the amino and carboxyl halves of Msg (25,26). In Western blot analysis, the carboxyl fragment was recognized more frequently by serum specimens than the amino fragment but did not distinguish between HIV-positive patients and healthy donors. An enzyme-linked immunosorbent assay (ELISA) using the carboxyl fragment showed significantly higher antibody levels in HIV-negative patients with *Pneumocystis* pneumonia (PCP) compared with healthy controls, but it could not distinguish among HIVpositive, PCP-positive and HIV-positive, PCP-negative patients and controls.

We recently developed three overlapping recombinant fragments of Msg that span the length of *P. jiroveci* Msg and used these antigens to measure serum antibodies by Western blot in healthy blood donors and HIV patients (27). The data showed significant differences in the frequency of reactivity to the Msg constructs, not only between the two groups but also between PCP-positive and PCP-negative patients with HIV.

Although Western blot is a valuable serologic technique, its major limitation is that it is not quantitative. ELISA overcomes this problem and is better suited for population surveys. We developed an ELISA using recombinant Msg fragments as antigens and analyzed antibody responses of healthy blood donors and HIV-positive patients (including PCP-positive and PCP-negative patients). We present three different analyses of the ELISA data and compare the results to those obtained by Western blot.

Materials and Methods

Serum samples as well as the demographic and clinical information about the human participants in this study were described in detail in our earlier report (27). That report also describes how recombinant Msg fragments were cloned, expressed, and purified and how Western blot was performed.

Enzyme-linked Immunosorbent Assay

Serum specimens were tested against the following antigens: recombinant Msg fragments; *Escherichia coli* extract expressing the pET vector without insert as a vector control; tetanus toxoid (TT) as a positive control; phosphate-buffered saline (PBS) without antigen (negative control). A standard serum sample, obtained from a healthy donor with known reactivity to MsgA, MsgB, and MsgC in Western blot, is run on each plate as a control. Duplicate wells of a 96-well plate were coated with antigen (1 μg/mL, 100 μL/well in PBS pH 7.4 overnight. The plates were washed in wash buffer (PBS with 0.05% Tween-20) and blocked with blocking buffer (wash buffer with 5% nonfat milk) (200 μL/well) for 2 h at room temperature. The plates were washed again, and human serum diluted 1/100 in blocking buffer was added to each well (100 μL/well). The plates were rocked overnight at 4°C, washed in wash buffer, and horseradish peroxidase (HRP)-labeled goat anti-human immunoglobulin (Ig) G (heavy and light chains) was added to each well (100 μL/well at 1/5,000 dilution in blocking buffer). HRP-labeled S-protein was used on each plate as a positive control and to correct for antigen loading. The plates were incubated at room temperature for 1 h, washed, and developed by adding 3,3′,5,5′-tetramethylbenzidine substrate (100 μL/well). Color was allowed to develop for 4 min, the reaction was stopped by adding 100 μL of 0.18 mol/L H_2SO_4 to each well, and the plates were read at a wavelength of 450 nm.

Data were analyzed three different ways. In ELISA 1, the reactivity of each serum specimen to Msg was expressed as the ratio of reactivity to the pET vector (mean optical density [OD] $Msg_{test\ serum}$ – mean OD $PBS_{test\ serum}$) / (mean OD $pET_{test\ serum}$ — mean OD $PBS_{test\ serum}$). In ELISA 2, the reactivity to Msg was expressed as the percent reactivity to TT for each serum: (mean OD $Msg_{test\ serum}$ — mean OD $PBS_{test\ serum}$) / (mean OD $TT_{test\ serum}$ — mean OD $PBS_{test\ serum}$) x 100. In ELISA 3, the reactivity to Msg was expressed as percent reactivity of the standard serum: (mean OD $Msg_{test\ serum}$ – the mean OD $PBS_{test\ serum}$) / mean OD $Msg_{standard\ serum}$ – the mean OD $PBS_{standard\ serum}$) x 100. Variations in assay results using the control serum were measured for MsgA and MsgC on a per-plate basis (n = 6, coefficients of variation [CV] 3%–5%for MsgA and 3.6%–7% for MsgC), a daily basis using two plates (n = 12, CV 3.3%–5.8% for MsgA and 4.8%–7.4% for MsgC), and an overall basis (across 4 days with two plates per day) (n = 48, CV 8.7% for Msg A and 13.3% for MsgC).

Statistics

Geometric means and 95% confidence intervals (95% CI) of observed ELISA measurements were obtained by patient category and antigen status. Before analysis, ELISA measurements were log-transformed to approximate normality. Weighted least squares regression analysis of variance (ANOVA) was carried out for each ELISA measurement separately to test the equality of ELISA means between PCP-positive, PCP-negative, HIV, and donor categories, adjusted for categorically modeled antigen level (A, B, C). The weighting values were the inverse of the within-category ELISA variances. The means of PCP-positive, PCP-negative, and HIV groups were compared to the mean of blood donors by calculating a post hoc linear contrast of individual estimates. The model included interactions between antigens and patient categories to allow for possible differences in the effect of patient status on mean values of ELISA among antigen levels. Significance was judged as $p < 0.05$, unless stated otherwise.

The association between Western blot positivity and ELISA was investigated by logistic regression in which Western blot (dichotomous ±) was related to continuously measured ELISA, adjusted for categorically modeled antigen level (A, B, C) and patient group (PCP-positive, PCP-negative, HIV, donor). Associations were obtained by calculating the odds of Western blot positive versus negative results for increasing ELISA reactivity, ranging from low (25th percentile) to high (75th percentile). From the same analysis, odds ratios (ORs) measuring associations between Western blot positivity and patient status (donor versus PCP-positive, PCP-negative, HIV) were calculated at midpoints of ELISA 1, 2, and 3, equal to 2.7, 16, and 35, respectively. ORs for the combined PCP groups, i.e., blood donor versus HIV, were obtained by comparing the mean of PCP groups to mean of the donor group, calculated from a linear contrast of the individual group effects. Significance was judged by obtaining 95% CI of ORs. A CI that does not include 1 indicates a significant association (two-sided p = 0.05) between the odds of Western blot reactivity when ELISA results are high versus low, or between blood donors and patient groups at the midpoint of ELISA measurements.

Spearman correlation coefficients were obtained by measuring associations between ELISA and CD4 and viral counts in HIV patients. All statistical analyses were performed by using the SAS statistical analysis system (SAS for Windows, Version 8.2, SAS Institute, Cary, NC.)

Results

Analysis of HIV-Positive and Blood Donor Serum Specimens

The reactivity of serum antibodies from HIV patients and healthy blood donors to MsgA, MsgB, and MsgC was compared by three different ELISA analyses (Table 1). Irrespective of the method of analysis, HIV-positive patients had significantly higher levels of antibody than healthy blood donors to each of the Msg fragments ($p < 0.001$), even though the range of ELISA values overlapped between the two groups (Figure). In blood donors, antigen reactivity was hierarchical (MsgC > MsgA > MsgB) for all methods of analysis. Similar results were seen for HIV patients in ELISA 1 and 2, but in ELISA 3 reactivity to MsgB was higher than to MsgA or MsgC (not significant).

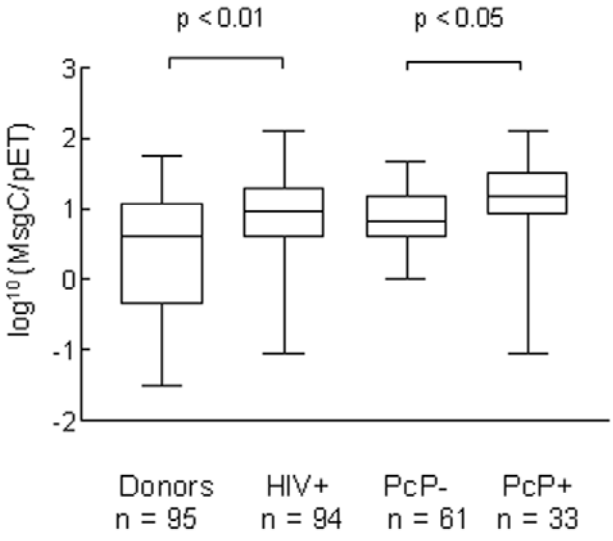

Figure. Antibody reactivity of healthy blood donors (donors); HIV-positive; PCP-positive, HIV-positive; and PCP-negative, HIV-positive patients to human *Pneumocystis* major surface glycoprotein C (MsgC) by enzyme-linked immunosorbent assay 1 (ratio to pET), showing the range, 25% and 75% confidence intervals, and median of the data. Data were log-transformed to approximate normality.

To determine if the increased reactivity of the HIV serum samples could be accounted for by increased antibodies in the PCP-positive patients as a consequence of infection with *Pneumocystis*, we compared the reactivity of HIV-positive, PCP-negative and HIV-positive, PCP-positive patients with that of blood donors (Table 1, Figure). Irrespective of the ELISA method chosen, the reactivity of PCP-negative serum samples was higher than that of blood donor serum samples for each of the Msg fragments ($p < 0.01$), indicating that PCP status alone could not account for the increased antibody reactivity found in the HIV cohort.

Table 1. Geometric mean values and 95% CI of ELISA by blood donor, PCP-positive, PCP-negative, and HIV status for each antigen[a]

ELISA	Antigen	Blood donor, n = 95 (95% CI)	PCP+, n = 33 (95% CI)	PCP–, n = 66 (95% CI)	HIV+ (PCP+, PCP–), n = 94 (95% CI)
1 (n = 189)	A	1.2 (0.8 to 1.8)	6.8 (4.1 to 11.3)	6.0 (4.6 to 7.7)	6.2 (4.9 to 7.9)
	B	1.0 (0.7 to 1.4)	3.7 (2.4 to 5.8)	2.9 (2.3 to 3.6)	3.2 (2.5 to 3.9)
	C	2.0 (1.2 to 3.1)	13.0 (7.9 to 21.4)	7.5 (6.0 to 9.3)	9.1 (7.3 to 11.4)
2 (n = 189)	A	9.0 (6.3 to 12.7)	31.2 (22.0 to 44.2)	28.9 (21.2 to 39.5)	29.7 (23.6 to 37.4)
	B	4.2 (2.7 to 6.6)	16.2 (11.5 to 22.7)	15.0 (11.9 to 18.9)	15.4 (12.8 to 18.6)
	C	21.4 (15.0 to 30.7)	57.8 (48.1 to 69.3)	39.1 (31.7 to 48.2)	44.9 (38.5 to 52.2)
3 (n = 189)	A	18.6 (10.5 to 32.9)	73.5 (53.0 to 102.1)	73.8 (54.1 to 100.7)	73.7 (58.6 to 92.7)
	B	8.2 (4.0 to 16.6)	102.6 (73.0 to 144.2)	103.8 (83.0 to 129.7)	103.4 (86.1 to 124.2)
	C	22.1 (12.6 to 38.6)	120.1 (99.8 to 144.5)	81.2 (66.7 to 98.9)	93.2 (80.5 to 107.9)

[a]CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; PCP, *Pneumocystis* pneumonia.

Comparison of PCP-Negative and PCP-Positive Serum Specimens

PCP-positive patients had significantly higher reactivity to MsgC than PCP-negative patients by all three methods of analysis ($p < 0.05$, $p < 0.01$, and $p < 0.01$ for ELISA 1, 2, and 3, respectively). Reactivity to MsgA or MsgB did not significantly differ between the populations by any method of analysis.

In ELISA 1, MsgC elicited the highest reactivity in both PCP-positive and PCP-negative groups, which was significant when compared to reactivity to MsgB ($p < 0.001$ in both groups) but not to MsgA (p nonsignificant in both groups). In ELISA 2, the recognition of MsgC by PCP-positive patients was significantly higher than that of MsgA or MsgB, and MsgA exhibited higher reactivity than MsgB ($p < 0.01$, all comparisons). Similarly, in PCP-positive patients, the highest reactivity in ELISA 3 was to MsgC, which was significant when compared to MsgA ($p < 0.01$) but not compared to MsgB ($p > 0.05$). In contrast to the other ELISA methods, the highest levels in the PCP-negative group were to MsgB, but this finding was not significant ($p > 0.05$).

Correlation between ELISA and Western Blot

To determine whether a correlation existed between ELISA values and reactivity by Western blot analysis, we compared data obtained by ELISA with Western blot results by logistic regression. As demonstrated by the adjusted ORs and 95% CI, a significant association was found between seropositivity by Western blot and higher ELISA values among HIV-positive patients ($p < 0.05$) but not among blood donors (Table 2). This association was found for reactivity to all Msg fragments and all ELISA methods, with the highest association being found for ELISA 3. Significant and positive associations were also found between Western blot reactivity and blood donor/HIV status at ELISA midpoints (geometric mean values shown by ELISA) (Table 3). Again, the highest value was found with ELISA 3.

Tables 2 and 3 show results of logistic regression analyses relating ELISA values and Western blot reactivity in PCP-positive and PCP-negative patients. A significant association was found between increasing ELISA 1 values and Western blot seropositivity in PCP-positive patients only ($p < 0.05$). Similar results were found for each Msg fragment in each patient group. Also, a significant association between increasing ELISA 2 levels and Western blot positivity in PCP-positive patients was found ($p < 0.05$). The relationship between ELISA 2 measurements and Western blot reactivity varied by Msg fragment in PCP-negative patients; a significant association was found for Msg C only ($p < 0.05$, OR = 16.9, 95% CI = 3.6 to 79.7). For ELISA 3, a significant OR was found for both patient groups ($p < 0.05$). Significant and positive associations were also found between Western blot reactivity and blood PCP status at ELISA midpoints (geometric mean values of ELISA) (Table 3), with the highest value found for ELISA 3. Spearman correlations were obtained between ELISA values and CD4+ cell counts or viral titer in PCP-positive and PCP-negative patients. No significant associations were noted (data not shown).

In our earlier study, PCP developed in 9 of the 61 PCP-negative patients after their serum samples had been obtained. None of these persons showed antibodies to MsgC by Western blot. These patients had a geometric mean antibody level in ELISA 1 o f 5.47 (95% CI 2.57 to 11.64) compared with a level of 7.88 (95% CI 6.25 to 9.94) in the 52 PCP-negative patients. Although mean serum levels were not significantly different among patient groups ($p > 0.05$), some trends were interesting. Patients in whom PCP developed subsequent to collecting their specimens had lower antibody levels to MsgC than the other patient groups.

Discussion

Using ELISA, we have compared blood donor and HIV-positive patient serum samples for antibody reactivity to recombinant fragments of *P. jiroveci* Msg. We analyzed the data by three independent methods and found that HIV-positive patients had significantly higher mean serum antibody levels to MsgA, MsgB, and MsgC than healthy blood donors by all methods of analysis. Furthermore, when the HIV-positive patients were separated on the basis of a prior documented episode of PCP, the PCP-positive patients had higher antibody levels to MsgC than the PCP-negative patients by all ELISA methods tested. These results differ from published work (25,26) showing no difference in antibody levels between HIV-positive,

Table 2. Adjusted odds ratios (95% CI) measuring the associations between Western blot reactivity and ELISA values (25–75 percentile increase) among 189 study participants[a,b]

ELISA	Blood donor, n = 95 (95% CI)	PCP+, n = 33 (95% CI)	PCP–, n = 61 (95% CI)	HIV, n = 94 (95% CI)
1	1.1 (0.9 to 1.4)	5.1 (2.2 to 11.8)	1.4 (0.8 to 2.6)	2.7 (1.2 to 5.8)
2	1.1 (0.9 to 1.4)	20.4 (6.4 to 65.1)	3.6 (2.0 to 6.6)	8.6 (4.4 to 16.9)
3	1.0 (0.9 to 1.1)	11.6 (3.6 to 37.9)	7.4 (3.6 to 15.1)	9.3 (4.7 to 18.5)

[a]CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; PCP, *Pneumocystis* pneumonia.
[b]Odds ratios were obtained by using logistic regression. Positive associations were found between Western blot reactivity and increasing ELISA. Based on 95% confidence intervals, the odds of Western blot reactivity were significantly related ($p < 0.05$) to increasing ELISA 1, 2, and 3 among PCP+ patients and HIV patients across all antigens. For PCP– patients this result was true for ELISA 2 and 3 only. No significant results were found for the blood donor group.

Table 3. Adjusted odds ratios (95% CI) measuring the associations between Western blot reactivity and participant status (blood donor versus PCP-positive, PCP-negative, HIV) among 189 study participants[a,b]

ELISA	Midpoint	Blood donor: PCP+ (95% CI)	Blood donor: PCP– (95% CI)	Blood donor: HIV (95% CI)
1	2.7	3.2 (1.6 to 6.7)	2.2 (1.4 to 3.5)	2.7 (1.7 to 4.3)
2	16	5.9 (2.5 to 14.3)	3.2 (2.0 to 5.3)	4.3 (2.6 to 7.4)
3	35	10.0 (2.9 to 33.3)	9.1 (4.4 to 20.0)	9.4 (4.6 to 19.3)

[a]CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; PCP, *Pneumocystis* pneumonia.
[b]Odds ratios measuring the association between Western blot reactivity and participant status were calculated at the midpoints of ELISA measurements over all antigens. Based on 95% confidence intervals, blood donors had significantly increased odds of Western blot reactivity compared to each patient group ($p < 0.05$) for each ELISA.

PCP-positive patients; HIV-positive, PCP-negative patients; and healthy controls to recombinant fragments of Msg. The discrepancy in results could be due to differences in study populations, Msg preparations, and methods (28).

At the onset of the study, we decided on three independent methods of analysis of the data in an attempt to standardize the assay. Each method of analysis provides different information about the results of the ELISA, and each method has strengths and weaknesses. The first analysis involved comparing the response to recombinant Msg fragments with the response to protein expressed from the pET vector without insert. The protein expressed from the sequences of the pET vector is an integral part of each Msg recombinant tested, and therefore this method of analysis allows measurement of Msg-specific immune reactivity corrected internally for any reactivity to the vector-derived sequences. The second method of analysis was a comparison of the reactivity to Msg with that of a standard antigen, tetanus toxoid, chosen because it is likely that most adults have been vaccinated against tetanus toxoid and are likely to react with this protein in assay. While this method is consistent when analyzing individual serum samples, one potential problem with this analysis is that the antibody response to tetanus toxoid varies from person to person. This variation means that, when a population is studied, the range of values obtained by this method can be quite large. The third method of analysis was comparing the test serum reactivity to that of a standard serum. Our standard serum was chosen because of clear reactivity to all Msg fragments tested in Western blot analysis. However, this serum responded weakly to MsgB in ELISA. Our choice of standard serum is still valid and is useful for comparing the reactivity to any one of the fragments in different populations. However, the use of this method to compare responses to different Msg fragments must be treated with caution. An alternative approach would be to use a pool of serum samples from several donors chosen for reactivity to the individual Msg fragments.

We have previously shown that MsgB was the most common fragment recognized by blood donors and HIV patients (27), and a significant difference was found in the frequency of recognition of MsgB between donors and HIV-positive patients. In contrast to these results, this study has shown that MsgC is the antigen with the highest level of reactivity in both the healthy and HIV-positive populations tested. This apparent discrepancy between assays is probably due to the nature of the epitopes recognized in the different assays, since the recombinant antigens were reduced and denatured during testing by Western blot analysis but not in ELISA. The lack of strict concordance in recognition of Msg fragments in ELISA and Western blot has also been seen by other groups (15,26) However, we did find a significant association between ELISA antibodies and Western blot seropositivity in HIV-positive patients but not in blood donors. Using regression analysis, we calculated ORs measuring the association of Western blot positivity and study participant (blood donor vs. HIV) status at the midpoint of each ELISA. These results showed a significantly greater likelihood of being Western blot–positive among blood donors than HIV patients. These data can be explained by several factors. First, 84% of our blood donors, but only 66% of the HIV patients ($p = 0.003$), responded to at least one of the three Msg fragments. Second, blood donors have consistently lower ELISA values than HIV patients. Third, blood donors exhibit larger standard deviations than HIV patients in their ELISA values, suggesting they have a more heterogeneous antibody response in terms of affinity for Msg epitopes. The association between Western blot positivity and ELISA values is probably not due to the PCP-positive patients' response, as PCP-negative patients also showed an association in two of the three ELISA methods.

Despite their lower Western blot seropositivity rate, HIV patients had significantly higher mean serum antibody levels to MsgA, MsgB, and MsgC than healthy blood donors by both methods of ELISA analysis. That HIV-positive patients should have higher levels of antigen-specific reactivity than healthy blood donors is surprising. One possible explanation is that HIV-positive patients may come into contact with *Pneumocystis* more frequently or for longer periods of time before the organism is cleared from the lungs (1). While this exposure to *Pneumocystis* may not result in overt PCP, the immune system likely takes longer to clear the organisms from the lung, allowing responses to new or subdominant epitopes to develop. In contrast, these responses would be absent or diminished in

healthy populations, who would clear *Pneumocystis* from the lungs more quickly. Therefore, the higher response in HIV-positive patients could be due to the accumulated responses to multiple epitopes, whereas the responses in healthy blood donors may be limited to immunodominant epitopes. An alternative explanation may be that the elevated levels of reactivity seen in HIV-positive serum are specific for the IgG isotypes and that the elevated levels of the isotype profile of Msg-specific antibodies may be skewed in healthy persons and HIV-positive patients.

This study shows that PCP-positive patients have higher antibodies to MsgC than PCP-negative patients by all ELISA methods. Furthermore, nine HIV patients in whom PCP developed after their serum specimens had been obtained and who did not have antibodies to MsgC by Western blot (27) had lower levels of antibodies to MsgC in this study. Overall, a hierarchy appeared to exist in the level of serum antibodies to MsgC by ELISA: highest in PCP-positive patients, next highest in PCP-negative patients, and lowest in blood donors.

Humoral immunity has long been thought to have little role in host defenses against *Pneumocystis* because PCP develops in many patients despite preexisting antibodies to the organism (8). Yet considerable evidence, mainly from animal models, now suggests that B cells contribute to these host defenses (2). Also of interest is that some serologic studies have shown decreased antibody levels or production before a person acquires PCP or increased antibody levels after a patient recovers from PCP (6,8,23,29–34). Our data suggest that analysis of the immune reactivity to Msg fragments, MsgC in particular, may be important in understanding differences between patient populations and may lead to identifying epitopes linked to protection from or susceptibility to PCP in populations at risk.

Acknowledgments

This work was supported by the Medical Research Service, Department of Veterans Affairs, and by the Public Service contracts AI 75319 and AI 25467 and grant RO1 HL64570 from the National Institutes of Health.

Dr. Daly holds a Ph.D. in molecular genetics, biochemistry, and microbiology. His research interests have included viral immunology and viral and tumor immunology. He has been a research associate at the University of Cincinnati since 1998, where he has been studying immune responses to *Pneumocystis*.

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Vancomycin Susceptibility within Methicillin-resistant *Staphylococcus aureus* Lineages

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Methicillin-resistant *Staphylococcus aureus* (MRSA) with reduced vancomycin susceptibility vancomycin-intermediate *S. aureus* (VISA) has been reported from many countries. Whether resistance is evolving regularly in different genetic backgrounds or in a single clone with a genetic predisposition, as early results suggest, is unclear. We have studied 101 MRSA with reduced vancomycin susceptibility from nine countries by multilocus sequence typing (MLST), characterization of SCC*mec* (staphylococcal chromosomal cassette *mec*), and *agr* (accessory gene regulator). We found nine genotypes by MLST, with isolates within all five major hospital MRSA lineages. Most isolates (88/101) belonged to two of the earliest MRSA clones that have global prevalence. Our results show that reduced susceptibility to vancomycin has emerged in many successful epidemic lineages with no clear clonal disposition. Increasing antimicrobial resistance in genetically distinct pandemic clones may lead to MRSA infections that will become increasingly difficult to treat.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major problem around the world, causing hospital-acquired infections and, more recently, infections in the community (1,2). The glycopeptides, particularly vancomycin, have been the mainstays of therapy for MRSA, and the emergence of resistance to these agents is of great concern.

The first *S. aureus* with reduced vancomycin susceptibility (vancomycin MIC ≥8 μg/mL) was isolated in 1997 (3,4), and similar isolates have since been discovered in several countries. These vancomycin-intermediate *S. aureus* (VISA) isolates are relatively rare; a recent review found 21 VISA described in the literature (5). However, strains of *S. aureus* have been described that are vancomycin-susceptible by conventional testing but have a subpopulation of resistant cells. These heterogeneous VISA (hVISA) are more common; reports from around the world indicate that 0.5%–20% of MRSA are heteroresistant (5). The clinical importance of hVISA is debatable, but evidence shows that they are precursors of VISA, and they have been implicated in treatment failure in deep-seated infections (6,7).

A study of early VISA strains that used multilocus sequence typing (MLST) and analysis of the SCC*mec* region suggested that they were all descended from the New York/Japanese (8,9) pandemic MRSA clone (10); the first high-level vancomycin-resistant isolates that have acquired the *vanA* gene cassette from enterococci are also members of this clone (F. Tenover, pers. comm.). Researchers have suggested that isolates of the New York/Japanese pandemic MRSA clone may be predisposed to become vancomycin resistant, perhaps because of loss-of-function mutations in the *agr* (accessory gene regulator) gene (11). We analyzed the genetic backgrounds of a geographically diverse sample of VISA and hVISA to investigate the evolutionary history of such strains.

Materials and Methods

We collected 101 isolates of MRSA with reported heterogeneous or homogeneous resistance to vancomycin (MIC ≥ 8 mg/L) from China (n = 1), France (31), Japan (2), Norway (14), Poland (13), Sweden (1), United Kingdom (28), and the United States (11). Antimicrobial susceptibility tests were performed by the agar dilution method of the National Committee for Clinical Laboratory Standards. Isolates were described as VISA if they fulfilled the three criteria adopted by the Centers for Disease Control and Prevention, that is, broth microdilution vancomycin MIC of 8 to 16 mg/L, MIC ≥ 6 mg/L on E-test, and growth on brain-heart infusion agar containing 6 mg/L vancomycin (12). Isolates with heterogeneous resistance to vancomycin were confirmed by using population analysis profiling followed by measuring the area under the curve (PAP-AUC), as described previously (13). The prototypic

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hVISA strain MU3 was used as a standard, and isolates with an AUC ≥0.9 compared to MU3 were described as hVISA.

MLST was performed as described previously (10). The seven housekeeping gene sequences were compared to known alleles in the MLST database (available from http://www.mlst.net), and the resulting allelic profiles (which define sequence types, STs) were used to interrogate the databases for matches within records of the 988 isolates held there. The MLST databases contain molecular and epidemiologic data on *S. aureus* isolates from carriage and disease, including examples of all major MRSA clones (10). Data from this study were added to the *S. aureus* MLST database, and the entire dataset was analyzed by using the BURST algorithm to assign isolates to clonal complexes (CCs), which are lineages containing genetically related isolates (sharing 100% genetic identity at ≥5/7 loci used). Polymerase chain reaction (PCR) analysis of the *ccr* (chromosomal cassette recombinase) and *mec* (methicillin resistance) regions was performed to discriminate the four main SCC*mec* types (I–IV) on the basis of combinations of the two regions. Conventional PCR was used to detect SCC*mec* I–III by using the primers described in Ito et al. (14) and SCC*mec* IV by using those described by Daum et al. (15). These results were confirmed using the multiplex method of Oliveira et al. (16). Detection of *agr* subgroups I–IV was performed by PCR of the region surrounding *agrD*, which codes for an autoinducing peptide, according to the method of Peacock et al. (17).

Results and Discussion

The results are shown in the Table. PAP-AUC values for the isolates varied from 0.9 to 3.01 and 91/101 isolates were designated hVISA on the basis of a PAP-AUC value ≥0.9. Nine isolates were designated as VISA.

From the genotyping results, strains were divided into clonal complexes, which can be subdivided according to sequence type (ST) and SCC*mec* differences. The clonal complexes CC5, CC8, CC22, CC30, and CC45 represent the five pandemic MRSA lineages that have been previously described (10). Our results show that hVISA has arisen in all five of these pandemic clones and that VISA has so far developed in CC5 and CC8. The three most common MRSA clones present in the United Kingdom (EMRSA-3, EMRSA-15, EMRSA-16) (18) are included within these lineages, and reduced vancomycin susceptibility has been identified in all of these clones. All lineages displayed resistance to multiple antimicrobial classes, and only the new oxazolidinone linezolid was active against all strains.

Only *agr* subgroups (alleles) I and II were found in isolates in this study with 7/9 VISA and 57/92 hVISA having

Table. Characteristics of study isolates with reduced vancomycin susceptibility[a]

Genotype								Antimicrobial susceptibility[*†]				
CC	ST	SCC*mec*	Clonal type	*agr* type	Vancomycin resistance phenotype (no. of strains)	PAP-AUC	Country of origin	Lzd	Syn	Gen	Cip	Rif
5	5	I	EMRSA-3	II	hVISA (1)	0.98	UK	S	S	R	R	R
				I	VISA (1)	1.9	USA	S	S	R	R	S
5	5	II	New York/Japanese	II	hVISA (10)	0.97–1.23	Japan, Sweden, France, Poland, UK, USA, Norway	S	S	S/R	S/R	S/R
				I or II	VISA (3)	1.4–1.92	USA	S	S	S	R	S/R
5	5	IV	Pediatric	I or II	hVISA (3)	1.19–1.32	UK	S	S	S	S/R	S/R
5	5	NT		I	VISA (1)	1.44	France	S	S	R	R	R
8	8	I		II	hVISA (3)	0.92–1.32	France, UK, Norway	S	S	R	R	R
8	8	II	Irish-1	II	hVISA (3)	1.04–1.2	France, USA, Norway	S	S	S/R	R	S/R
8	8	IV	EMRSA-4, -6	I	hVISA(11)	0.94–1.24	France, USA	S	S/R	S/R	R	S/R
22	22	IV	EMRSA-15	I or II	hVISA (7)	0.9–1.25	UK	S	S	S	R	S/R
25	25	NT		I	hVISA(1)	1.13	UK	S	S	R	R	R
30	36	II	EMRSA-16	II	hVISA (3)	0.92–1.17	UK	S	S	R	R	R
45	45	II		I	hVISA (1)	1	USA	S	S	R	R	S
8	239	I or II	Brazilian/ Portuguese	I or II	hVISA (10)	0.9–1.22	France, Poland, China, Norway, UK	S	S	R	S/R	S/R
				I	VISA (3)	1.44–3.01	France, Poland, UK	S	S/R	R	R	S/R
8	239	NT		I	hVISA (1)	0.92	France	S	S	R	R	R
8	246	NT		I	hVISA (1)	1.13	Norway	S	S	R	R	R
8	247	I	Iberian	I	VISA (1)	1.57	UK	S	S	R	R	R
				I or II	hVISA (37)	0.9–1.33	France, Poland, UK, Norway	S	S	R	R	S/R

[a]S, susceptible; R, resistant; NT, nontypeable; PAP-AUC, population analysis profiling followed by measuring the area under the curve; Lzd, linezolid; Syn, synercid; Gen, gentamicin; Cip, ciprofloxacin; Rif, rifampin; CC, clonal complex; ST, sequence type; EMRSA, methicillin-resistant *Staphylococcus aureas* found in the United Kingdom (UK) ; hVISA, heterogeneous vancomycin-intermediate *S. aureus*; USA, United States of America.

agr I. Within the 14 clones in this study, the proportion of isolates with particular *agr* alleles was variable. The presence of both *agr* I and *agr* II among VISA/hVISA, even in genetically similar isolates, suggests that the genes for the *agr* system are horizontally transferred. Sakoulas et al. reported an association of *agr* II with the development of vancomycin resistance (11). Our results show that VISA/hVISA also emerged in strains with *agr* I.

Molecular analyses of VISA isolates to date have focused on isolates from the United States and Japan, and results have indicated that all strains belong to the New York/Japanese MRSA clone. In our study, we found that hVISA isolates have emerged from every lineage that has produced pandemic MRSA clones, and VISA isolates have emerged in two of five lineages, in all likelihood from hVISA precursor isolates.

Increasing drug resistance in clones that are multidrug resistant and adapted to spread and cause serious disease can do much damage in the modern hospital environment. We have shown that reduced vancomycin susceptibility has emerged in genetically and phenotypically diverse MRSA clones throughout the world. This finding suggests that vancomycin resistance has the potential to become a widespread problem in MRSA strains already resistant to multiple antimicrobial agents.

This work was funded by the Wellcome Trust. M.C.E. is a Royal Society University Research Fellow.

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Syndromic Surveillance in Public Health Practice, New York City

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The New York City Department of Health and Mental Hygiene has established a syndromic surveillance system that monitors emergency department visits to detect disease outbreaks early. Routinely collected chief complaint information is transmitted electronically to the health department daily and analyzed for temporal and spatial aberrations. Respiratory, fever, diarrhea, and vomiting are the key syndromes analyzed. Statistically significant aberrations or "signals" are investigated to determine their public health importance. In the first year of operation (November 15, 2001, to November 14, 2002), 2.5 million visits were reported from 39 participating emergency departments, covering an estimated 75% of annual visits. Most signals for the respiratory and fever syndromes (64% and 95%, respectively) occurred during periods of peak influenza A and B activity. Eighty-three percent of the signals for diarrhea and 88% of the signals for vomiting occurred during periods of suspected norovirus and rotavirus transmission.

Two recent phenomena have contributed to widespread interest in monitoring nonspecific health indicator data to detect disease outbreaks early. The first is heightened concern about bioterrorism, particularly the ability of public health agencies to detect a large-scale bioterrorist attack in its early stages. The second is the proliferation of electronic databases in healthcare settings. Initially designed to facilitate billing, health information systems capture an increasingly rich array of clinical detail. Recent advances in information technology make extracting, transmitting, processing, and analyzing these data feasible for public health purposes. The emergency department surveillance system we describe is an early prototype of what may become a standard component of modern public health surveillance.

In New York City, emergency department chief complaint surveillance evolved out of the public health response to the September 11, 2001, World Trade Center attacks (1). When this labor-intensive effort ended, the New York City Department of Health and Mental Hygiene (DOHMH) began intensively recruiting hospitals capable of providing emergency department visit data in electronic formats. We describe the methods and chief results from the first 12 months of experience with this electronic system.

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Materials and Methods

Data Transmission and Processing

Data files are transmitted to DOHMH 7 days per week, either as attachments to electronic mail messages or through direct file transfer protocol (FTP). Half of participating hospitals have automated the transmission process. Data processing and analysis are carried out on a laptop computer that can be operated either through the DOHMH local area network or through remote dial-up, which facilitates weekend and holiday analysis. Each morning, an analyst retrieves the files, inspects them for quality and completeness, and saves them for processing and analysis in SAS (version 8, SAS Institute Inc., Cary, NC). If a file is not received by 10:00 a.m., the analyst contacts hospitals to obtain missing data. The analysis is typically completed by 1 p.m.

Data files contain the following information for all emergency department patient visits logged during the previous midnight-to-midnight 24-hour period: date and time of visit, age in years, sex, home zip code, and free-text chief complaint. Additionally, some hospitals provide either a visit or medical record number. No other personal identifiers are included. Files arrive in several formats, most commonly as fixed-column or delimited ASCII text. Data are read and translated into a standard format, concatenated into a single SAS dataset, verified for completeness and accuracy, and appended to a master archive.

Syndrome Coding

Emergency department patient visits are categorized into exclusive syndromes based on the patient's chief complaint, a free-text field that captures the patient's own

description of his/her illness. We developed a SAS algorithm that scans the chief complaint field for character strings assigned to a syndrome. The coding algorithm is designed to capture the wide variety of misspellings and abbreviations in the chief complaint field. If the chief complaint was blank or uninformative (e.g., "EVAL," "TRIAGE") the record was omitted. If it contained a word or phrase from a single category it was coded for that syndrome, i.e., "SHORTNESS OF BREATH" or "SOB" appearing alone would indicate the respiratory syndrome. If the chief complaint contained words or phrases from multiple categories, it was coded according to the following hierarchy: common cold > sepsis/dead on arrival > respiratory > diarrhea > fever > rash > asthma > vomiting > other visits. The hierarchy attempts to place each chief complaint into a single, specific syndrome (Table 1). Chief complaints containing text strings such as, "cold," "sneeze," "stuffy," or "nasal" are coded as cold and excluded to increase the specificity of the respiratory category for illnesses other than viral rhinitis. The two syndromes of particular interest for bioterrorism surveillance are the respiratory and fever syndromes in persons ≥13 years of age. Children are excluded due to their high rates of febrile and respiratory illnesses and to limit the number of false signals generated. Respiratory and fever syndromes in children are examined by graphic and CUSUM analyses with SaTScan performed on an ad hoc basis. We monitor the diarrhea and vomiting syndromes in all ages in an effort to detect gastrointestinal outbreaks that may be due to contamination of food or water.

Statistical Methods

Separate analyses are carried out for each syndrome-age category of interest to look for citywide temporal increases and clustering by either hospital location or patient's home zip code. The intent of the system is to detect moderate- to large-scale events and not single cases. The term "signal" hereafter refers to a statistically significant aberration (2).

Citywide Temporal Analysis

The primary method for evaluating citywide trends in syndrome visits is an adaptation of the one-dimensional temporal scan statistic (3–5) to a prospective setting with daily analyses and a variable-length window consisting of the last 1, 2, or 3 days. This adaptation is a special case of the prospective space-time scan statistic (6). The ratio of syndrome visits to nonsyndrome (other) visits during the most recent 1, 2, or 3 days is compared to a 2-week baseline. The choice of a maximum window length is flexible. We set this length to 3 days to be able to detect sharp 1-day spikes as well as more gradual increases over 2 or 3 days. Using SaTScan version 2.1.3 (7), we calculate a likelihood ratio statistic that reflects the difference between the observed data and what would be expected under the null hypothesis (no temporal trend). Significance of citywide signals is derived through Monte Carlo hypothesis testing by ranking these likelihoods within a distribution of similarly calculated likelihood ratios from 999 random datasets. Our p values are adjusted for the multiple comparisons inherent in both the multiple window lengths evaluated as well as for running daily prospective analyses (6).

Spatial Clustering Analysis

The spatial scan statistic (7), originally developed for the retrospective analyses of chronic diseases, has been adapted for infectious disease surveillance (8). This approach requires comparing the observed to the expected number of cases in each geographic area. In cancer epidemiology, the expected cases can be well-approximated by using the underlying (age-adjusted) population, but this approach cannot detect infectious disease outbreaks when using healthcare utilization data, as higher rates of illness and emergency-department utilization in some areas of the city are found at baseline. To control for these purely spatial differences, expected counts of syndrome visits are derived from each area's history, rather than from the underlying census population. Since rapidly emerging outbreaks are of particular interest, we take the data from the observed cases from the last day and compare them with data from a 14-day baseline period, ending 2 days earlier (i.e., a 1-day gap is left between the baseline and the date on which spatial clustering is being evaluated). The expected number of visits in a geographic area is calculated as follows:

$$E = T \bullet \frac{S_{city}}{T_{city}} \bullet \frac{S_{baseline} \,/\, T_{baseline}}{S_{city\ baseline} \,/\, T_{city\ baseline}}$$

where T is the total visits in the geographic area on the date being evaluated, S_{city} and T_{city} are the number of syndrome and total visits citywide on this date, $S_{baseline}$ and $T_{baseline}$ are the number of syndrome and total visits in the geographic area during the 14-day baseline, and $S_{city\ baseline}$ and $T_{city\ baseline}$ are the corresponding citywide numbers.

For both the citywide temporal and spatial clustering analyses, the most likely cluster for the observed dataset is determined by using the SaTScan software to calculate the likelihood

$$\left(\frac{O_{in}}{E_{in}}\right)^{O_{in}}\left(\frac{O_{out}}{E_{out}}\right)^{O_{out}}$$

where O is the observed count for the syndrome and E is the expected count. Significance is derived from ranking this likelihood ratio among 999 randomized datasets by

Table 1. Syndrome coding and hierarchy

Syndrome	Includes	Excludes
Common cold	Nasal drip, congestion, stuffiness	Chest congestion, sore throat
Sepsis	Sepsis, cardiac arrest, unresponsive, unconscious, dead on arrival	
Respiratory	Cough, shortness of breath, difficulty breathing, croup, dyspnea, bronchitis, pneumonia, hypoxia, upper respiratory illness, chest congestion	Cold
Diarrhea	Diarrhea, enteritis, gastroenteritis, stomach virus	
Fever	Fever, chills, flu, viral syndrome, body ache and pain, malaise	Hay fever
Rash	Vesicles, chicken pox, folliculitis, herpes, shingles	Thrush, diaper and genital rash
Asthma	Asthma, wheezing, reactive airway, chronic obstructive airway disease	
Vomiting	Vomiting, food poisoning	

using Monte Carlo simulations. A significant signal is defined by a p value < 0.01, so that for each syndrome we would expect, on average, one false signal every 100 days. This threshold set the frequency of signal investigations at a sustainable level.

Dissemination of Results

Each day's analyses are reviewed with a medical epidemiologist, and a report consisting of graphs and a brief summary is distributed by electronic mail to program staff. If a signal investigation is performed, a more detailed report is prepared and made available by the next day. An external report summarizing citywide trends is also distributed daily to state and regional health officials, the New York City (NYC) Office of Emergency Management, police departments, and fire departments. Hospital-specific, confidential reports are shared quarterly with participating emergency departments, comparing their facility to overall citywide trends.

Signal Response

The investigation of a spatial syndromic signal begins with descriptive review of the emergency department visits included in the signal. Syndrome observed and expected values by hospital are reviewed to focus the investigation at the hospital(s) contributing the largest number of excess cases. A line list of patients with their chief complaints is produced, along with summary statistics for age, sex, and zip code. Chief complaints that are wrongly coded, such as "denies fever" coded as fever syndrome, are noted, and the coding algorithm is amended. For a subset of emergency departments, an electronic or paper interim chief complaint log covering visits since midnight can be obtained to determine whether the number of syndrome visits remains elevated. Phone calls are made to emergency department and other hospital staff to alert them to unusual disease patterns and to ask whether they have noted an increase in the frequency of syndrome visits or admission of seriously ill patients. Signals of continuing concern are further investigated by field staff conducting chart reviews, patient interviews, and onsite discussions with clinicians.

Results

During the surveillance period (November 15, 2001, to November 14, 2002), 2.5 million patient visits were recorded from 39 participating emergency departments. The citywide average number of visits was 6,780/day (mean per emergency department = 174; range 36–460).

The median daily proportion of emergency departments that transmitted data in time for analysis was 95%. This proportion ranged from 63% on a day when the central electronic mail system for 11 public hospitals was inoperable to 100% on 104 of the 365 surveillance days. Timeliness of reporting was higher for the 21 hospitals that used automated data transmission (median 100% reported on time) than for the 18 hospitals that relied on manual transmission (median 81% reported on time; Wilcoxon test $p < 0.001$). Data were also more complete on weekdays (median 97% reported on time) than on weekends and holidays (median 86%, Wilcoxon test $p < 0.001$).

The chief complaint field was blank or uninformative in 4.1% of records. The proportion of visits with missing or uninformative chief complaint did not vary significantly by age, sex, or day of week but was concentrated at nine emergency departments where from 11% to 30% of records had missing or uninformative chief complaint each day. Among records with chief complaints, 0.3% were missing age, and 2.0% were missing zip code.

Table 2 shows the distribution, by syndrome category, of the 2,374,131 (96%) emergency department visits for which chief complaint was informative. Marked differences were found in the distribution of chief complaints by age. The key syndromes (respiratory, fever, diarrhea, and vomiting) accounted for 42% of visits among children age ≤12 years compared to only 12% among those ≥13 years of age.

Citywide Signals

Respiratory and Fever Syndromes

A citywide signal in the respiratory syndrome was first detected on December 25, 2001, and citywide signals for both fever and respiratory syndromes occurred over the following 6 days. This signal provided the earliest indication of communitywide influenza activity in New York

Table 2. Distribution of emergency department visits by hierarchical syndrome category (valid chief complaints)

Syndrome (in order of hierarchy)	% all ages (n = 2,374,131)	% age 0–12 y (n = 556,065)	% age 13–39 y (n = 399,895)	% age 40–64 y (n = 248,290)	% age ≥65 y (n = 99,567)
Common cold	2.0	5.8	1.0	0.9	0.5
Sepsis/dead on arrival	0.5	0.2	0.2	0.6	1.8
Respiratory	8.0	13.3	4.4	6.7	11.9
Diarrhea	1.6	3.6	0.9	0.9	1.0
Fever	6.5	20.0	2.7	2.0	1.9
Rash	2.0	4.1	1.6	1.2	0.6
Asthma	3.3	4.9	2.6	3.3	1.9
Vomiting	2.7	4.7	2.3	1.7	2.1
Other	73.5	43.7	84.4	82.4	78.3

City for the 2001–02 season (Figure 1). This series of signals began 2 weeks before increases in positive influenza laboratory isolates were noted and 3 weeks before sentinel physician increases in influenzalike illness were reported. Retrospectively, these emergency department signals coincided by illness onset date with a sharp increase in positive laboratory tests for influenza A (Figure 1).

Overall, 14 (64%) of 22 citywide respiratory signals and 21 (95%) of 22 citywide fever signals occurred during periods of peak influenza A and B activity. Three other respiratory signals—an isolated signal in late November 2001 and a pair of signals in September 2002—coincided with an increase in asthma visits, and a series of five signals in October 2002 occurred during a period of steadily increasing respiratory visits. Only one isolated fever signal occurred outside of the influenza season, during August 2002.

Diarrhea and Vomiting Syndromes

Diarrhea and vomiting signals occurred during three periods of communitywide gastrointestinal illness activity. In November 2001, we observed sharp increases in emergency department visits and repeated signals for diarrhea and vomiting syndrome among both children and adults (Figure 2). Concurrently, DOHMH received reports of institutional outbreaks whose characteristics were consistent with norovirus. In one instance, a calicivirus was isolated. A second major increase in gastrointestinal illness occurred during February and March of 2002 and was most pronounced among children under age 5 with diarrhea. Local hospital-based virology laboratories were queried and reported an increase in requests for rotavirus tests and positive results. Finally, a series of diarrhea and vomiting signals was again noted in November 2002 before widespread reports of institutional outbreaks. During the ensuing 6 weeks, norovirus was identified in stool specimens from 3 (75%) of 4 emergency department patients and 18 (69%) of 26 persons identified through five separate outbreak investigations.

Overall, 15 (83%) of 18 diarrhea signals and 21 (88%) of 24 vomiting signals occurred during these three outbreak periods. Three additional isolated vomiting signals and one series of three consecutive diarrhea signals of unknown cause occurred in July 2002.

Spatial Signals

Respiratory and Fever Syndromes

A total of 25 hospital-based respiratory or fever signals and 18 zip code–based signals occurred during the 12-month period, in which we would have expected only 7 (730 analyses-days x 0.01, Table 3). The number of respiratory and fever spatial signals occurring in October 2001 through March 2002 (when the citywide incidence of respiratory and fever visits was higher), 21 signals/183 surveillance days, was no different than the number occur-

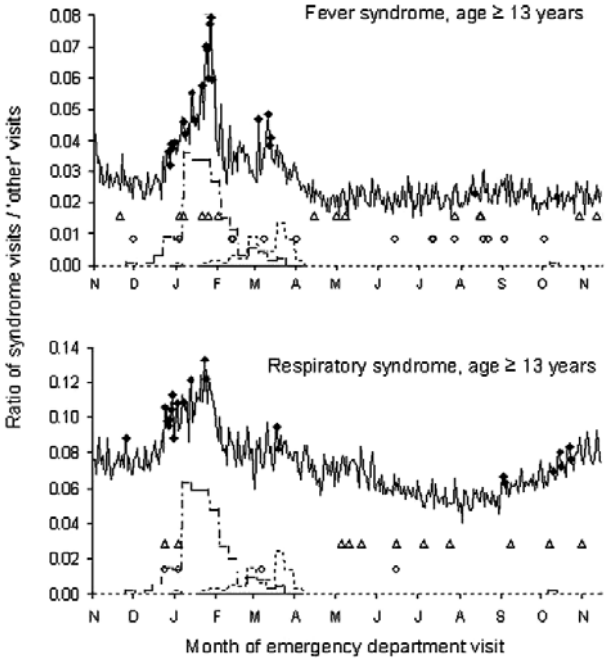

Figure 1. Trends in emergency department visits for fever and respiratory syndromes, New York City, November 1, 2001–November 14, 2002. Plots show the daily ratio of syndrome visits to other (noninfectious disease) visits. ♦, citywide signal; Δ, spatial signal by hospital; ❍, spatial signal by patient's home zip code; – · – , influenza A; ------- , influenza B isolates (weekly number identified in New York City residents by World Health Organization collaborating laboratories).

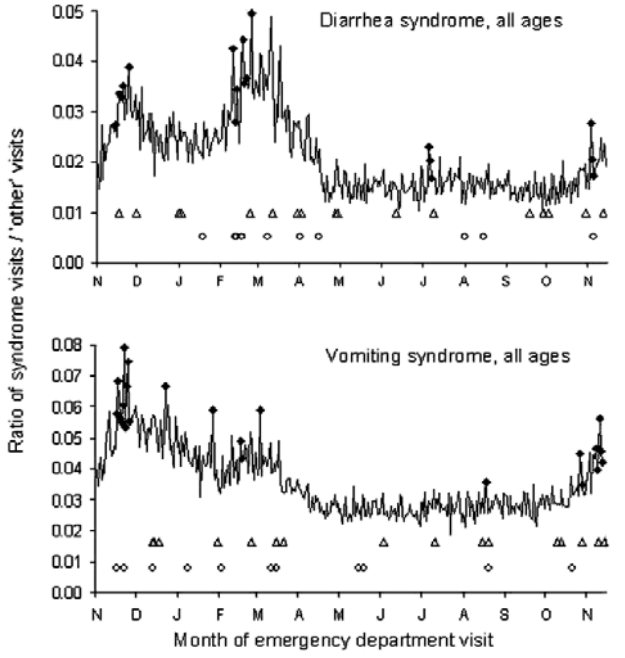

Figure 2. Trends in emergency department visits for diarrhea and vomiting syndromes, New York City, November 1, 2001–November 14, 2002. Plots show the daily ratio of syndrome visits to other (noninfectious disease) visits. ♦ = citywide signal; Δ = spatial signal by hospital; ❍ = spatial signal by patient's home zip code.

ring during the rest of the year (22 signals/182 surveillance days, χ^2 p = 0.9). No spatial signal persisted for >1 day in the same area. Investigations of these spatial signals showed no sustained increase in visits and no illnesses indicating bioterrorism. No localized natural outbreaks were detected, although few diagnostic specimens were collected and tested in response to spatial signals. No large respiratory outbreaks were reported through traditional surveillance during this period.

Diarrhea and Vomiting Syndromes

A total of 34 hospital-based diarrhea or vomiting signals and 21 zip code–based signals were found during the 12-month period (Table 2). Signals occurred more frequently between mid-October and mid-April when citywide incidence of gastrointestinal illness was high (36 signals/183 surveillance days) compared to the rest of the year (19 signals/182 surveillance days, χ^2 p = 0.02). Several moderate- to large-sized institutional gastrointestinal outbreaks were reported to the DOHMH but not detected through syndromic surveillance. One reported gastrointestinal outbreak involved schoolchildren, who went to a nonparticipating hospital's emergency department. Retrospective review of data from this outbreak showed that it would have been detected had this hospital participated during that period.

Discussion

In its first year of operation, the emergency department syndromic system identified communitywide increases in gastrointestinal illness in all ages consistent with norovirus (9), an increase in diarrheal illness among young children consistent with rotavirus, and the arrival of epidemic influenza. DOHMH used this information to alert the medical community of the arrival of these pathogens, something it had not been able do in a timely fashion previously. The system also detected many single-day spatial signals suggestive of illness clusters; however, none of these signals were verified as outbreaks. Several foodborne and institutional gastrointestinal outbreaks occurred in New York City during the surveillance period and were not identified by our emergency department surveillance system. None of the other outbreaks reported through traditional means during this period gave a simultaneous syndromic signal, and unreported outbreaks were probably likewise missed. A major limiting factor of syndromic surveillance using emergency visits for mild or prodromal illness is that adults with gastroenteritis or mild respiratory symptoms usually do not seek medical care in emergency departments.

The operational strengths of the emergency department syndromic surveillance system we describe include its ease of initial setup and relatively low cost of maintenance (direct DOHMH costs estimated at $130,000 per year for 40 hospitals and population >8,000,000). Cooperation from hospital staff aided this effort considerably. Forty-five hospitals in New York City, covering an estimated 80% of emergency department visits, currently participate in the surveillance system. Achieving fully automated, standardized, and encrypted data transmission has been a slow process but remains a priority.

Public health authorities in general (10) and the New York City DOHMH in particular (11) have a legal mandate to conduct surveillance for outbreaks and are covered by specific provisions in the Health Insurance Portability and Accountability Act. However, good public health practice requires that steps be taken to minimize the privacy risk to persons and institutions. These steps include collecting the minimum amount of identifiable data necessary (e.g., age rather than date of birth), encrypted data transfer, and protocols for limiting access to potentially identifiable information.

One key attribute of syndromic surveillance systems is timeliness. We selected chief complaint for categorizing emergency department visits into syndromes because it is available in electronic format within hours of the patient's arrival. The accuracy of chief complaint as an indicator of patient illness has been evaluated and was shown in one study to have good agreement for the syndromes of respiratory and gastrointestinal illness (12) and in another study

Table 3. Summary of signals based on the temporal and spatial scan statistics ($p \leq 0.01$) November 15, 2001–November 14, 2002

Analysis/syndrome	No. signals	Mean observed/expected cases in cluster	Mean relative risk	Mean excess cases in cluster
Citywide temporal analysis				
Respiratory (age ≥13 y)				
1-day	1	283/224	1.3	59
2-day	8	770/673	1.1	96
3-day	13	1,107/984	1.1	123
Fever (age ≥13 y)				
1-day	3	225/166	1.4	59
2-day	6	418/332	1.3	85
3-day	13	591/497	1.2	94
Diarrhea				
1-day	3	129/83	1.6	46
2-day	6	283/219	1.3	64
3-day	9	423/349	1.2	73
Vomiting				
1-day	6	232/175	1.3	58
2-day	6	448/359	1.2	88
3-day	12	741/635	1.2	106
Hospital spatial analysis				
Respiratory (age ≥13 y)	11	71/44	1.6	27
Fever (age ≥13 y)	14	32/16	2.0	16
Diarrhea	17	26/12	2.2	14
Vomiting	17	38/20	1.9	18
Zip code spatial analysis				
Respiratory (age ≥13 y)	4	56/33	1.7	23
Fever (age ≥13 y)	14	18/6	3.0	12
Diarrhea	10	17/6	2.8	11
Vomiting	11	23/9	2.6	14

to be equally sensitive and specific as discharge diagnosis for acute respiratory illness (13). Both studies used coding algorithms that differ from those of DOHMH as well as each from each other. Standardization of coding algorithms would facilitate comparing system results. CDC, the Department of Defense Global Emerging Infections System, and representatives of model systems have created ICD9-based standard syndromes that may serve as a template for chief complaint coding (14).

The desire to quickly detect outbreaks (especially those due to bioterrorism) has also influenced our approach to aberration detection algorithms. The methods we have adapted are designed to detect increases in syndromes that occur within 1 to 3 days, rather than provide greatest sensitivity for detecting outbreaks that have been building for a week or more (15). Debate exists on whether a bioterrorist attack would be first detected by an astute clinician diagnosing severe illness or by syndromic surveillance detecting focal or widespread prodromal illness. Our system has thus far not detected a localized outbreak, and whether the spatial clusters represent true localized outbreaks, statistical noise, or clustering due to other causes remains unknown. Answering this question will require accumulated experience with true localized outbreaks, more intensive investigation of spatial signals, or simulation studies with outbreak scenarios and "spiked" datasets.

Some critics have highlighted the challenges of investigating syndromic signals while preserving the advantage of time they afford (16). Analytic methods and investigation protocols must be designed so they do not overburden public health agencies. Our experience suggests several lessons in this regard: 1) the number of syndromes and analyses used increases the number of signals that need to be evaluated, 2) determining the signal threshold is as much operational as statistical: thresholds must be set at a sustainable level for public health investigation and reflect changing levels of concern for outbreaks, and 3) sustained geographic signals are rare. A stepwise approach to field investigations is a practical way of limiting the costs and burden of the system.

The NYC DOHMH system is unique in that the operational, response, and research components are integrated within a health department. The staff members who analyze data are the same as or work closely with those who perform signal investigations. Knowledge of the data and system operational aspects is invaluable for understanding signals and following up with emergency departments.

Surveillance systems that use existing electronic data can provide timely information about the health of the population at low cost and with minimal effort on the part of data providers. Our syndromic surveillance system has helped detect communitywide outbreaks and reassure the

public during high-profile public events. It can be readily adapted to other uses, and we have explored this potential by tracking emergency department visits for asthma, dog bites, heat-related illness, suicide, and drug overdoses. We have used the system to find cases of rash illness, measles, anthrax powder hoaxes, putative spider bites, and botulismlike illness. Nevertheless, syndromic surveillance systems are essentially "smoke detectors" and call for prompt investigation and response if they are to provide early warning of outbreaks. Syndromic surveillance should be viewed as an adjunct to, not a replacement of, traditional disease surveillance. For many, if not all, state and local public health departments, the emphasis of bioterrorism preparation should be on hiring well-trained public health professionals with responsibilities beyond bioterrorism.

Acknowledgments

We thank the medical and information technology staff of participating New York City emergency departments and the Department of Health and Mental Hygiene Syndromic Surveillance Team: Joel Ackelsberg, Sharon Balter, Melanie Besculides, Jane Greenko, Jessica Hartman, Deborah Kapell, Marcelle Layton, Jennifer Leng, Kristi Metzger, Michael Phillips, Vasudha Reddy, Carla Rodriguez, Richard Rosselli, Linda Steiner, and Polly Thomas.

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Multidrug-resistant Tuberculosis in Central Asia

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Multidrug-resistant tuberculosis (MDR-TB) has emerged as a major threat to TB control, particularly in the former Soviet Union. To determine levels of drug resistance within a directly observed treatment strategy (DOTS) program supported by Médecins Sans Frontières in two regions in Uzbekistan and Turkmenistan, Central Asia, we conducted a cross-sectional survey of smear-positive TB patients in selected districts of Karakalpakstan (Uzbekistan) and Dashoguz (Turkmenistan). High levels of MDR-TB were found in both regions. In Karakalpakstan, 14 (13%) of 106 new patients were infected with MDR-TB; 43 (40%) of 107 previously treated patients were similarly infected. The proportions for Dashoguz were 4% (4/105 patients) and 18% (18/98 patients), respectively. Overall, 27% of patients with positive smear results whose infections were treated through the DOTS program in Karakalpakstan and 11% of similar patients in Dashoguz were infected with multidrug-resistant strains of TB on admission. These results show the need for concerted action by the international community to contain transmission and reduce the effects of MDR-TB.

Tuberculosis (TB) has increased substantially in many parts of the former Soviet Union, particularly in those areas most affected by economic decline and failing health infrastructures (1). In addition to resurgent TB, significant proportions of multidrug-resistant TB (MDR-TB) have been demonstrated in small pockets where drug -susceptibility surveys have been conducted, and these areas have been termed "hot spots" (2,3). This article reports the results of a drug-susceptibility survey conducted by Médecins Sans Frontières (MSF) in collaboration with local ministries of health, in northwestern Uzbekistan and northern Turkmenistan in Central Asia. These regions, in addition to experiencing a substantial economic crisis, are facing a severe water shortage and the desiccation of the Aral Sea (4).

A TB treatment program based on the directly observed treatment strategy (DOTS) (5) recommended by the World Health Organization was introduced progressively into three regions south of the Aral Sea by the humanitarian medical aid organization, Médecins Sans Frontières. These regions, the Republic of Karakalpakstan, and Khorezm Oblast in Uzbekistan and Dashoguz Velayat in Turkmenistan (Figure 1), have a total population of approximately 4 million people. Implementation of DOTS started in 1998 and was completed with full coverage by late 2003. In 2002, more than 8,000 patients were registered and treated under DOTS from the three regions. Facilities for culturing sputum and

Figure. Aral Sea area, Uzbekistan and Turkmenistan.

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drug susceptibility testing were not available in the area at the time of the survey.

From the start of DOTS implementation, >10% of those treated remained smear-positive. This finding, combined with a dearth of information on drug resistance in Central Asia, prompted the initiation of a drug-susceptibility survey with the aim of determining levels of resistance to both first- and second-line anti-TB drugs and, in particular, the levels of MDR-TB.

The current survey was conducted in two of the regions covered by DOTS, the Republic of Karakalpakstan in Uzbekistan and Dashoguz Velayat in Turkmenistan. Although adjacent, these regions are separated by an international border. Key characteristics of these regions and countries are given in Table 1; the two regions differ in terms of total case reporting rate and in economic status.

Methods

Study Design

The survey was designed on the basis of recommendations given in the World Health Organization/International Union Against Tuberculosis and Lung Disease **(**WHO/IUATLD) guidelines for surveillance of drug-resistant TB (8). It was designed to be a cross-sectional survey of smear-positive pulmonary TB patients in whom DOTS TB treatment had been initiated in four districts in the Autonomous Republic of Karakalpakstan in Uzbekistan and four districts in Dashoguz Velayat in Turkmenistan. Three of the districts in each region were selected because they had the longest running DOTS programs in the region. The fourth district in each region was selected as the newest district implementing DOTS before initiation of the survey. When the survey was initiated, 7 of the 17 districts in Karakalpakstan were implementing DOTS TB treatment, as were 7 of the 9 districts in Dashoguz. Although selection of districts in which to conduct the survey was not random, all smear-positive patients whose TB was diagnosed in DOTS laboratories (regardless of previous TB treatment status) from July 2001 in the selected districts were sequentially requested to participate in the survey. Patients were excluded if they refused to undergo DOTS treatment or if they did not reside in one of the districts chosen in the study. Patients defined as "chronic," that is, patients whose TB was not resolved after at least two courses of DOTS treatment, were not eligible to be included in the study. Smear positivity was defined as at least one sputum sample reading >10 bacilli/100 fields in a sputum smear by direct microscopy.

The objectives of the study were described to each patient, and written informed consent was obtained before an additional sputum sample was collected for the study. All patients were informed that the results would not be available to either themselves or their treating physician and would not affect their treatment. At the time of the survey, no opportunity was available for patients to be treated with second-line drugs under DOTS-Plus. To ensure patient confidentiality, sputum samples and clinical information forms were encoded with a unique survey identification number.

MDR-TB is defined as resistance to at least isoniazid and rifampin. On the basis of estimated levels of 10% and 20% MDR-TB for new and retreatment cases, sample sizes were calculated to be 81 for new cases (10%±6%, 95% confidence limit [CL]) and 100 for re-treatment cases (20%±7%, 95% CL). Because approximately 50% of patients with positive smears have been previously treated, a sample size of 100 was chosen for each category in both regions, for a total of 400 patients. Recruitment continued until the desired sample size was reached or surpassed in both categories and in both regions.

Demographic and Clinical Information

Each patient who consented to participate in the survey was interviewed by the admitting doctor in each TB facil-

Table 1. Key characteristics of the two regions and countries included in the survey[a]

	Uzbekistan	Turkmenistan
Population (6)	25,256,000	4,834,000
GDP per capita (6)	U.S.$ 2,333	U.S.$ 5,269
Health expenditure per capita (6)	U.S.$ 86	U.S.$ 286
Estimated TB incidence rate (all cases) (7)	92/100,000/y	84/100,000/y
Estimated smear-positive case detection (7)	44%	68%
	Karakalpakstan	Dashoguz
Population (2001 estimated)[b]	1,549,761	1,041,372
DOTS implementation	1998 (pilot districts)–2003 (full coverage)	2000 (pilot districts)–2002 (full coverage)
DOTS case notification rate (all cases)	482/100,000/y	213/100,000/y
DOTS case notification rate (smear-positive cases)	192/100,000/year	89/100,000/year
% of smear-positive cases previously treated (2002)	55	53
Success rate (new smear-positive cases registered in 2001)	68%	82%

[a]GDP, gross domestic product; TB, tuberculosis; DOTS, directly observed treatment strategy.
[b]Source: ministries of health in each region.

ity. A clinical information form was developed, based on recommendations by the WHO and IUATLD (8), to collect basic demographic, socioeconomic, and medical information. Particular attention was paid to questions that would clarify previous TB treatment for each patient. Questions were included about previous hospitalization and about which anti-TB drugs the patient may have taken. Previous TB treatment assessed by the interview was then compared to the patient's previous TB treatment status as determined through the functioning DOTS program. Any discrepancies were then investigated and clarified by reinterviewing the patients and physicians who treated their condition. In addition, for patients from Karakalpakstan only, medical records from the previous Soviet system of TB treatment were checked for previous TB treatment.

New patients were defined as those who had received no or <1 month of antituberculosis drug treatment before diagnosis. Retreatment patients were all those previously treated with TB drugs for ≥1 month. Data on the HIV status of patients with TB are not available in this area.

Transport of Sputum Samples and Laboratory Testing

Because no local capability for culture and drug-sensitivity testing exists, sputum samples were transported directly to a supranational reference laboratory (SRL) in Europe. Sputum specimens were shipped from both regions separately either weekly or biweekly throughout the survey. Samples were first transported to the regional laboratories in each region by car. They were then flown to the capital cities in Uzbekistan and Turkmenistan; they were then transported as international air cargo to Frankfurt, Germany, and on to Hamburg and the SRL in Borstel, Germany. All international regulations pertaining to the transport of infectious material were followed.

Sputum samples were cultured, and drug-sensitivity testing was performed by the German SRL in Borstel. Strains were tested for drug sensitivity to the five first-line drugs used in the DOTS program in the Aral Sea area, namely, isoniazid, rifampin, pyrazinamide, ethambutol, and streptomycin. In addition, drug-susceptibility testing for three second-line drugs was performed; susceptibility to prothionamide was tested on all strains, whereas susceptibility to capreomycin and ofloxacin was tested on all strains, excluding the first 45 sent from both countries (24 from Uzbekistan and 21 from Turkmenistan, 11% of strains). Strains from a small, randomly drawn sample of patients with MDR-TB (20 isolates) and patients whose strains were susceptible to antimicrobial drugs (10 isolates) were later tested for resistance to kanamycin.

Primary isolation and culture of mycobacterial isolates followed standard recommendations, where specimens were decontaminated by NALC-NaOH and added to two solid media; Löwenstein-Jensen and Stonebrink ad MGIT960TB (Becton-Dickinson Microbiology Systems, Cockeysville, MD) (9). For all strains, drug-susceptibility testing was performed on Löwenstein-Jensen media by the proportion method. If growth was insufficient, drug-susceptibility testing was performed by using the modified proportion method in BACTEC 460TB (Becton-Dickinson). The SRL participates in annual quality control programs within Germany and within the WHO and Centers for Disease Control and Prevention (CDC) SRL network. In addition, an internal quality control system for all techniques and media was used.

Statistical Analyses

All clinical and laboratory data were entered into a database by using EpiInfo (6.04, CDC, Atlanta, GA). Chi-square analysis was used for comparisons of proportions. Logistic regression analysis was performed to identify variables independently associated with MDR-TB (SPSS version 10.0, SPSS Inc., Chicago, IL). Cluster sampling was taken into account through use of the Fleiss quadratic approximation in calculating 95% CL for the proportions of MDR-TB in the sample. Separate design effects were calculated for each region.

Results

Recruitment of Patients

Patient recruitment began in July 2001 in both regions and was completed by the end of January 2002 in Karakalpakstan and March 2002 in Dashoguz. In total, 441 patients were enrolled in the study, 213 from Dashoguz and 228 from Karakalpakstan. Overall, 76% of eligible patients were recruited in Dashoguz and 68% in Karakalpakstan. The main reasons patients were not enrolled were the following: refusal to participate (13% in Karakalpakstan and 3% in Dashoguz), patient default before treatment (8% in both Karakalpakstan and Dashoguz), and logistic constraints in the timely collection and transport of sputum samples (9% in Karakalpakstan and 2% in Dashoguz). Other reasons for nonparticipation in the study included the following: unable to produce an additional adequate sputum sample (5% in Dashoguz), patient imprisonment after diagnosis (3% in Dashoguz), and patient transfer out of the program after diagnosis. The relative biases introduced by these reasons for nonparticipation are unknown; however, the high rate of refusal to participate in Karakalpakstan was mostly due to inadequate patient communication in the early stages of the survey and is therefore unlikely to have affected the representativeness of the sample.

Laboratory Culture Results

All 441 sputum samples were sent to SRL in Borstel,

Germany. *Mycobacterium tuberculosis* was cultured from 416 samples (213 from Karakalpakstan and 203 from Dashoguz). Overall, 12 (2.7%) of the 441 samples were found to be contaminated, and an additional 11 (2.5%) could not be cultured. Other than *M. tuberculosis*, two other strains of *Mycobacterium* (one *M. bovis* and one *M. fortuitum*) were grown.

Demographics of the Study Population

For the purposes of analysis, only patients for whom a valid culture for *M. tuberculosis* was obtained were included. The final sample consisted of 106 new patients and 107 retreatment patients in Karakalpakstan and 105 new cases and 98 retreatment cases in Dashoguz. Patients ranged in age from 11 years to 77 years, with a mean of 34 years and a median of 31 years. The mean age was slightly but nonsignificantly higher in retreatment patients. Overall, 60% of the patients in the study sample were male, with more men among retreatment cases than new cases (65% vs. 55%). Overall 53% of all patients with positive smears registered in the DOTS program in the year 2002 in Karakalpakstan and Dashoguz were male.

Considerably more single men than single women lived in both regions; overall, 19% of women and 40% of men were single. Approximately 70% of all patients, both men and women in both countries, reported at least 10 years of education. No women reported previous imprisonment, whereas 25% of men in Dashoguz and 36% of men in Karakalpakstan reported being in prison at some stage during their lives.

First-line Anti-TB Drug Resistance

Results on resistance to the five first-line drugs tested are given in Tables 2 and 3. In Karakalpakstan, the strains of 52% of new patients and 20% of retreatment patients were fully sensitive to all five first-line drugs. In Dashoguz, strains from 70% of new patients and 38% of retreatment patients were sensitive to the five first-line drugs. The most notable monoresistance (resistance to just one first-line drug) was seen for streptomycin in both regions (in 11% of all Karakalpakstan patients and in 14% of Dashoguz patients). Similarly, streptomycin showed the highest levels of drug resistance overall; strains from 58% of all patients in Karakalpakstan and from 37% of patients in Dashoguz were resistant to streptomycin. Major resistance to isoniazid also occurred, with 53% of all patients in Karakalpakstan and 31% in Dashoguz infected with resistant strains. Resistance to rifampin was closely correlated to multidrug resistance; only one patient had a strain that was resistant to rifampin without also being resistant to isoniazid.

Levels of MDR-TB were high; overall, 27% (95% CL 15% to 42%) of all positive smears from patients starting DOTS treatment were infected with MDR-TB strains in Karakalpakstan and 11% (95% CL 7% to 17%) in Dashoguz. The proportion of MDR-TB was higher in Karakalpakstan than in Dashoguz for both new and retreatment cases; 13% (95% CL 4% to 35%) versus 4% (95% CL 1% to 11%) for new cases and 40% (95% CL 21% to 62%) versus 18% (95% CL 11% to 28%) for retreatment cases.

Table 2. First-line anti-tuberculosis drug resistance results, Karakalpakstan, Uzbekistan[a]

	No. new cases (%)	No. retreatment cases (%)	Total no. (%)
Total tested	106	107	213
Any resistance	51 (48.1)	86 (80.4)	137 (64.3)
Monoresistance			
H only	3 (2.8)	7 (6.5)	10 (4.7)
R only	0	0	0
E only	0	0	0
S only	12 (11.3)	11 (10.3)	23 (10.8)
Z only	0	1 (0.9)	1 (0.5)
H and R resistance			
MDR	14 (13.2)	43 (40.2)	57 (26.8)
HR only	0	1 (0.9)	1 (0.5)
HRE only	0	0	0
HRS only	4 (3.8)	10 (9.3)	14 (6.6)
HRZ only	0	0	0
HRES only	6 (5.7)	19 (17.8)	25 (11.7)
HREZ only	0	0	0
HRSZ only	1 (0.9)	0	1 (0.5)
HRESZ	3 (2.8)	13 (12.1)	16 (7.5)
H + other resistance			
HE only	0	0	0
HS only	13 (12.3)	15 (14.0)	28 (13.1)
HZ only	1 (0.9)	1	2 (0.9)
HES only	2 (1.9)	4 (3.7)	6 (2.8)
HEZ only	0	0	0
HSZ only	1 (.9)	3 (2.8)	4 (1.9)
HESZ only	5 (4.7)	1 (0.9)	6 (2.8)
R + other resistance			
RE only	0	0	0
RS only	0	0	0
RZ only	0	0	0
RES only	0	0	0
RESZ only	0	0	0
Any drug resistance			
Any H resistance	39 (36.8)	74 (69.2)	113 (53.1)
Any R resistance	14 (13.2)	43 (40.2)	57 (26.8)
Any E resistance	16 (15.1)	37 (34.6)	53 (24.9)
Any S resistance	47 (44.3)	76 (71.0)	123 (57.7)
Any Z resistance	11 (10.4)	19 (17.8)	30 (14.1)

[a]H, isoniazid; R, rifampin; E, ethambutol; S, streptomycin; Z, pyrizinamide.

Second-line Anti-TB Drug Resistance

Resistance results to the three second-line drugs first tested are shown in Table 4. Of these three drugs tested on most strains, clinically significant resistance was shown only to prothionamide, with 16% resistance among MDR-TB cases in Karakalpakstan and 9% in Dashoguz. Kanamycin was tested on a randomly drawn sample of

Table 3. First-line anti-tuberculosis drug resistance results, Dashoguz, Turkmenistan[a]

	No. new cases (%)	No. retreatment cases(%)	Total no. (%)
Total tested	105	98	203
Any resistance	32 (30.5)	61 (62.2)	93 (45.8)
Monoresistance			
H only	6 (5.7)	8 (8.2)	14 (6.9)
R only	0	1 (1.0)	1 (0.5)
E only	0	0	0
S only	16 (15.2)	13 (13.3)	29 (14.3)
Z only	0	0	0
H and R resistance			
MDR	4 (3.8)	18 (18.4)	22 (10.8)
HR only	0	0	0
HRE only	0	0	0
HRS only	3 (2.9)	7 (7.1)	10 (4.9)
HRZ only	0	0	0
HRES only	1	6 (6.1)	7 (3.4)
HREZ only	0	0	0
HRSZ only	0	3 (3.1)	3 (1.5)
HRESZ	0	2 (2.0)	2 (1.0)
H + other resistance			
HE only	0	1 (1.0)	1 (0.5)
HS only	5 (4.8)	10 (10.2)	15 (7.4)
HZ only	0	1 (1.0)	1 (0.5)
HES only	1 (1.0)	6 (6.1)	7 (3.4)
HEZ only	0	0	0
HSZ only	0	3 (3.1)	3
HESZ only	0	0	0
R + other resistance			
RE only	0	0	0
RS only	0	0	0
RZ only	0	0	
RES only	0	0	0
RESZ only	0	0	0
Any drug resistance			
Any H resistance	16 (15.2)	47 (48.0)	63
Any R resistance	4 (3.8)	19 (19.4)	23
Any E resistance	2 (1.9)	15 (15.3)	17
Any S resistance	26 (24.8)	50 (51.0)	76
Any Z resistance	0	9 (9.2)	9

[a]H, isoniazid; R, rifampin; E, ethambutol; S, streptomycin; Z, pyrizinamide.

20 previously identified MDR-TB isolates and 10 non-MDR-TB isolates from two districts in Karakalpakstan. Two of the MDR-TB isolates showed resistance to kanamycin (10%), whereas none of the 10 non-MDR-TB strains showed resistance.

Factors Associated with MDR-TB

To investigate factors associated with the high rates of MDR-TB, a multivariable logistic regression analysis was conducted with MDR-TB as the dependent variable. The following factors were entered into the model: sex, previous TB treatment, previous imprisonment, unemployment, alcohol use, and region. Factors significantly predicting MDR-TB in the model were previous TB treatment, region, and female sex (Table 5). Although a large proportion of male patients reported previous imprisonment, this factor was not significant on either univariate or multivariable analysis.

Since previous TB treatment is the strongest factor associated with MDR-TB, the logistic regression was repeated, including only new patients from both regions. Both female gender (OR 7.8, 95% CL 1.7 to 36.3) and region (OR 3.8, 95% CL 1.2 to 12.4) remained significant predictors of MDR-TB.

Clearly, the most notable factor predicting MDR-TB is previous TB treatment. Most retreatment patients in both regions reported only one previous episode of treatment, 60% in Karakalpakstan and 66% in Dashoguz. In addition, most retreatment patients were not previously treated under DOTS, 83% in Karakalpakstan and 75% in Dashoguz. Of those that received previous DOTS treatment, 50% were new patients before their previous DOTS treatment. These patients had levels of MDR-TB similar to retreatment patients overall (Karakalpakstan, 3/8, 38%; Dashoguz, 2/15, 13%). Those that received DOTS treatment in addition to previous non-DOTS TB treatment had higher rates of MDR-TB (Karakalpakstan, 6/8, 75%; Dashoguz, 7/14, 50%).

Discussion

This survey has shown extremely high rates of MDR-TB in regions of two countries in Central Asia, although the confidence intervals are large because of the small sample size and cluster sampling. These findings, along with similar data from other regions, suggest that the former Soviet Union is one large hot spot for MDR-TB.

Because of logistic constraints, sampling was restricted to four districts implementing DOTS in each of the two regions. These districts were not randomly selected. However, as sampling was sequential, the final sample is representative of patients with positive smears whose cases were diagnosed in these districts. The DOTS strategy was implemented in the Aral Sea area, starting in the districts most affected by the environmental degradation. These districts are more economically deprived and therefore may have higher rates of TB incidence. However, we have no reason to suspect that the prevalence of drug resistance is different in neighboring districts. Drug resistance may well increase as patients are increasingly able to purchase drugs privately and use them sporadically. In addition, patients who refused DOTS treatment and those defined as having chronic TB were not included in the survey. These patients are more likely to have had previous erratic TB treatment and therefore may be more likely to harbor drug-resistant strains. If this were the case, then the figures presented here are an underestimate of the situation.

In this survey, careful attention was paid to the differentiation of new and retreatment patients. A retrospective

Table 4. Second-line drug resistance results

	New		Retreatment		MDR-TB[b]	
	Total	Resistant (%)	Total	Resistant (%)	Total	Resistant (%)
Karakalpakstan (Uzbekistan)						
Prothionamide	106	7 (7)	107	11 (10)	57	9 (16)
Capreomycin	88	1 (1)	101	1 (1)	56	1 (2)
Ofloxacin	88	2 (2)	101	4 (4)	56	1 (2)
Dashoguz (Turkmenistan)						
Prothionamide	105	1 (1)	98	4 (4)	22	2 (9)
Capreomycin	89	0	93	0	21	0
Ofloxacin	89	0	93	0	21	0

[a]All strains were tested for prothionamide; a representative subset of these were tested for both capreomycin and ofloxacin.
[b]MDR-TB, multidrug-resistant tuberculosis.

review of pre-DOTS medical records in Karakalpakstan showed only one misclassified patient, among the 213 included. As well, in the Aral Sea area DOTS program, all patients in whom active TB is diagnosed have the opportunity to receive DOTS treatment, regardless of previous treatment status. Thus, no motivation or incentive exists for patients or doctors to misrepresent their previous TB treatment status, as has been suggested elsewhere (10). Nevertheless, some misclassification is possible but is not expected to greatly alter the high proportions of MDR-TB seen among new patients, particularly in Karakalpakstan. These levels of MDR-TB indicate the likely transmission of multidrug-resistant strains.

Testing showed some second-line drug resistance, particularly for prothionamide, among MDR-TB strains. The regions in our study are poorer and more isolated than other parts of both countries and other areas in the former Soviet Union, which possibly spares them from the high rates of second-line drug resistance seen in other areas (11). Although not all strains were tested for second-line resistance, the sample is still representative because only the first 11% of strains (Karakalpakstan and Dashoguz, respectively) from patients sequentially recruited into the survey were not tested for capreomycin and ofloxacin, and no systematic differences were found between the strains from first patients and strains from latter patients.

The difference in levels of TB drug resistance between the two regions that are geographically adjacent but separated by a national border provides clues regarding the emergence of drug resistance. Since gaining independence from the former Soviet Union, most Central Asian states have experienced a substantial decline in healthcare services (12). In Uzbekistan, during the 1990s, the gross domestic product (GDP) declined considerably as has the percentage of GDP spent on health (13). Turkmenistan fared somewhat better financially because of its considerable oil and gas wealth; it has a per capita GDP almost double that of Uzbekistan (6), with a relatively stable percentage spent on health (14).

These declines in healthcare spending in both countries have resulted in intermittent shortages of most first-line anti-TB drugs. Local TB physicians in Karakalpakstan (Uzbekistan) estimate that before the DOTS program started, nearly 50% of patients had their treatment interrupted because of problems with drug supply. Additionally, because of the lack of drugs, patients were often requested to purchase drugs themselves after they left the hospital for the continuation phase at home. Many patients likely could not afford all drugs and therefore purchased what they could, resulting again in treatment interruptions. High streptomycin resistance attests to the widespread use of this popular injectable antimicrobial agent, often as a short monotherapy course. Although drug shortages have been reported over the last decade in Turkmenistan, they likely affected an overall lower percentage of patients, which explains the lower rate of drug resistance seen in Dashoguz.

The extent to which drug resistance existed before the collapse of the Soviet Union is unknown. The Soviet system hospitalized TB patients for long periods, with consequently high levels of interruption and default. In addition, treatment regimens using combinations of all first-line and some second-line drugs were not standardized (1). These conditions may have contributed to a baseline level of resistance from which the notable level of MDR-TB shown here has emerged. Most retreatment patients were not previously treated under DOTS; thus, our results cannot be attributed to the implementation of DOTS.

Elsewhere in the former Soviet Union, high rates of MDR-TB have been seen among prisoner populations (15). Although a high proportion of patients in our study reported previous imprisonment, no greater level of MDR-TB was seen among these patients. This finding suggests that MDR-TB is not confined to specific sectors of the population, such as prisoners, but is a problem affecting the general community. Of particular concern in this area is the high rate of out-migration attributable to worsening environmental and socioeconomic conditions (16; unpub. data, Médecins Sans Frontières, 2002), which can lead to international transmission of MDR-TB.

The finding of a greater risk for MDR-TB among women, independent of previous TB treatment status, is

Table 5. Factors associated with MDR-TB in univariate and multivariable logistic regression analysis[a]

Factor	Odds ratios (95% confidence limits)	
	Univariate analysis	Logistic regression
Previous TB treatment	4.5 (2.6 to 8.0)	5.1 (2.8 to 9.3)
Region (Karakalpakstan)	3.0 (1.8 to 5.1)	3.6 (2.0 to 6.4)
Female gender	1.3 (0.8 to 2.2)	2.0 (1.1 to 3.7)
Unemployment	2.3 (1.1 to 5.1)	2.1 (0.9 to 4.8)
Previous imprisonment	1.7 (0.9 to 3.0)	1.3 (0.6 to 2.8)
Alcohol use	0.9 (0.4 to 2.3)	0.7 (0.3 to 1.9)

[a]MDR-TB, multidrug-resistant tuberculosis.

important and confirms similar findings in Archangels Oblast in Russia (17) and Estonia (18). In the Aral Sea area, women make up slightly less than 50% of all patients with positive smears registered in the DOTS program. This statistic suggests a greater susceptibility to drug-resistant TB and warrants further research.

Clearly, such high rates of MDR-TB as seen in both Karakalpakstan, Uzbekistan, and Dashoguz, Turkmenistan, are a substantial threat to TB control. Standardized treatment with first-line, through drugs implementing the DOTS strategy, would be expected to result in poor outcomes for more than one fourth of patients with positive smears in Karakalpakstan (19) and would render the WHO target of 85% success unattainable. These patients will remain infectious for long periods, with the resultant risk of transmitting drug-resistant strains. WHO suggests that high levels of MDR-TB (>3% among new cases) warrant the direct management of MDR-TB to contain transmission and reduce the high incidence and costs of this disease (20).

DOTS treatment on its own may well stop the production of more MDR-TB, but it is unlikely to reduce high levels of existing drug resistance (21). Effective treatment of all cases of TB is required to prevent transmission. MDR-TB treatment is expensive and lengthy, and the pool of those with expertise treating MDR-TB is limited. A simpler, more affordable, and more effective treatment strategy is required; however, until this exists, patients require treatment with existing strategies. As a result of this survey, Médecins Sans Frontières has decided to launch a pilot DOTS-Plus MDR-TB treatment project in Karakalpakstan because the cost of inaction will be high.

Acknowledgments

We thank the following persons and organizations for their assistance during this survey: the patients; the medical, nursing, and laboratory staffs from the eight districts; the ministries of health in Dashoguz Velayat and the Republic of Karakalpakstan; medical and logistics staff of Médecins Sans Frontières; staff of the National Reference Centre for Mycobacteria, Borstel, Germany; and the airlines and customs organizations who facilitated the shipment of sputum samples.

Financial support for this study was provided by the World Health Organization.

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Antimicrobial Resistance in Commensal Flora of Pig Farmers

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We assessed the quantitative contribution of pig farming to antimicrobial resistance in the commensal flora of pig farmers by comparing 113 healthy pig farmers from the major French porcine production areas to 113 nonfarmers, each matched for sex, age, and county of residence. All reported that they had not taken antiimicrobial agents within the previous month. Throat, nasal, and fecal swabs were screened for resistant microorganisms on agar containing selected antimicrobial agents. Nasopharyngeal carriage of *Staphylococcus aureus* was significantly more frequent in pig farmers, as was macrolide resistance of *S. aureus* from carriers. Nongroupable streptococci from the throat were more resistant to the penicillins in pig farmers. The intestinal isolation of enterococci resistant to erythromycin or vancomycin was not significantly higher in pig farmers in contrast to that of enterobacteria resistant to nalidixic acid, chloramphenicol, tetracycline, and streptomycin. Prevalence of resistance in predominant fecal enterobacteria was also significantly higher in pig farmers for cotrimoxazole, tetracycline, streptomycin, and nalidixic acid. We determined a significant association between pig farming and isolation of resistant commensal bacteria.

Higher prevalence of antimicrobial-resistant bacteria in commensal flora contributes to the general increase and dissemination of bacterial resistance worldwide (1,2) and can be a source of resistance genes for respiratory pathogens such as *Streptococcus pneumoniae* (3) and intestinal pathogens such as *Shigella* (4) or *Salmonella* (5,6). Antimicrobial treatments are major factors for selection of resistance in the commensal flora of humans (7). Industrial animal farming is also associated with large-scale antimicrobial use (8), which leads to a high level of colonization of animals with antimicrobial-resistant bacteria that can then contaminate the food and, in turn, humans (9,10). Farmers are more likely to acquire enteric antimicrobial-resistant bacteria from food-producing animals, even if not treated with antimicrobial agents themselves (11–14). However, this link has never been quantitatively assessed. Antimicrobial resistance in nasal and pharyngeal commensal strains might possibly be affected in the same manner, and this hypothesis has also not been investigated. We thus designed an exposed-nonexposed epidemiologic study to determine the association between contact with animals in pig-raising farms and isolation of antimicrobial-resistant nasal, pharyngeal, and intestinal commensal microorganisms.

Methods

Participants

The study population was composed of members of the Mutualité Sociale Agricole (MSA), a health insurance system for workers in agriculture and related services. We identified pig farmers as an exposed group and nonfarmers (such as those working at banks or in insurance services) as a nonexposed group. The sample size was calculated according to results on the prevalence of antimicrobial resistance in the fecal flora of French residents (15) to ensure that, for most markers measured, detection of a 10% difference in the exposed group would be found with a power of 80% and an α risk of 5%. Pig farmers were chosen among those working in large, exclusively pig farms (>84 pigs) and contacted during the yearly MSA preventive medicine visits to obtain permission for participation. One pig farmer per farm was randomly selected to fill a panel of 20 in each of the seven major French porcine production areas.

One nonfarmer control, matched for sex, age, and county of residence, was selected for each pig farmer and approached similarly. Nonfarmers were not living or working on a farm, in a slaughterhouse, or in the pharmaceutical

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industry and were not living with someone who worked on a farm.

Persons included in the study were judged healthy by physical examination, had no gastrointestinal symptoms or throat pain at inclusion, and reported that they had not been hospitalized or taken antimicrobial agents within the previous month. All study participants were enrolled within 3 months. Study participants' antimicrobial use in the 6 months preceding the study was retrospectively estimated from the MSA reimbursement database and converted to defined daily doses, as described (16). In cases in which methicillin-resistant *Staphylococcus aureus* (MRSA) was isolated, participants were further interviewed for hospitalization and contacts with hospitalized patients and healthcare workers during the previous year, as described (17). Occurrence and type of contact with pigs and contact precautions used in farms were documented in pig farmers with a standardized questionnaire. This study was performed in agreement with legal and ethical French regulatory procedures.

Specimens Obtained

Study participants were asked to bring fresh stool samples in sterile, closed cups. A sterile cotton swab was immersed in the sample. No procedure was implemented to ensure that participants brought their own stool specimens. They likely did, however, since participants were contacted during the yearly MSA preventive medicine visits by the practitioner with whom they had an established confidential relationship. Nasal swabs were inserted (1 cm) successively in both nares and rotated three times for 10 to 15 s. Pharyngeal samples were obtained by firmly pressing a swab over the tonsils and the posterior pharyngeal wall, and avoiding touching the jaws, teeth, or gingival when withdrawing the swab. All swabs were extemporaneously squeezed in sterile brain-heart infusion broth (BioMérieux, Marcy-l'Etoile, France) with 10% glycerol, immerged in liquid nitrogen within 6 hours, and stored at –80°C until processing.

Detection of Microbial Isolates

One hundred microliter–aliquots of all broth samples were plated as follows. For nasal samples, isolation of *S. aureus* was performed on Chapman agar (BioMérieux). Antimicrobial susceptibility of one isolate per participant was determined by using the disk diffusion technique (18).

For the pharyngeal samples, isolation of *Streptococcus pneumoniae* and β-hemolytic streptococci was performed on 5% sheep blood Columbia agar; isolation of *Haemophilus influenzae* was performed on chocolate agar, *Staphylococcus aureus* on Chapman agar, and yeast on Chromagar (all BioMérieux). Isolation of antimicrobial-resistant nongroupable streptococci was performed on 5% sheep blood Columbia agar supplemented with nalidixic acid and colistin. Antimicrobial-resistant nongroupable streptococci were detected on the same medium, supplemented with ampicillin (4 mg/L) or erythromycin (1 mg/L). For feces, aliquots were plated on Chromagar, Cetrimide (Bio-Mérieux), and Chapman agar for detection of yeasts, *Pseudomonas aeruginosa*, and *S. aureus*, respectively. Detection of enterococci of any resistance phenotype and of those resistant to erythromycin was performed on Bile-Esculin-agar (BEA) (BioMérieux) free of antimicrobial agents or supplemented with 5 mg erythromycin/L, respectively. Detection of vancomycin-resistant enterococci (VRE) was performed on BEA supplemented with 10 mg vancomycin/L after an enrichment step of 18 hours in broth containing 1 mg vancomycin/L, as described (19,20). The mechanism of vancomycin resistance was determined by polymerase chain reaction analysis, as described (21). Carriage of resistant enterobacteria was detected by using two separate procedures, as described (22), with modifications. In the first, designed to explore the subdominant flora, 0.1 mL of broth was plated on Drigalski agar supplemented with ampicillin (10 mg/L), ceftazidime (2 mg/L), streptomycin (20 mg/L), kanamycin (20 mg/L), chloramphenicol (20 mg/L), tetracycline (10 mg/L), or nalidixic acid (50 mg/L), as described (15). *Escherichia coli* of known susceptibility were used as the control. One of 10 positive plates was selected for quality control, and one colony was selected for antimicrobial susceptibility testing. A study participant was defined as colonized in the subdominant fecal flora with enterobacteria resistant to a given antimicrobial agent when at least one colony grew from the plate containing the corresponding antimicrobial agent.

In the second procedure, designed to explore the predominant fecal flora, Drigalski agar plates without antimicrobial agents were spread with 0.1 mL of broth culture. Five colonies were randomly selected. Those identified as *E. coli* were tested for antimicrobial susceptibility. A study participant was defined as colonized in the predominant flora by *E. coli* resistant to a given antimicrobial agent when at least one resistant strain was recovered from the feces by using this second procedure.

Statistical Analysis

The prescribed defined daily doses of an antimicrobial agent and the number of participants for whom antimicrobial agents had been ordered within the previous 6 months were compared between pig farmers and nonfarmers by using the Student *t* test for matched data. Differences between groups for carriage of nasal, pharyngeal, and fecal microbial species were analyzed by calculating matched prevalence ratios (PR) (23). For comparing antimicrobial-resistant phenotypes of *S. aureus*, nongroupable streptococci, *E. coli*, enterococci, and enterobacteria from pig

farmers and nonfarmer carriers, nonmatched PR were used, since these comparisons were performed on subgroups composed of only the carriers of the species with resistant clones that we examined. (For instance, rates of carriage of resistant enterobacteria were composed from subgroups of those actually carrying enterobacteria.) Because this analysis was performed only for carriers, a comparison in terms of age, sex, and location was performed to assess that pig farmers and nonfarmer carrier subgroups were comparable for these variables. Frequency of co-resistance to ampicillin, streptomycin, and trimethoprim-sulfamethoxazole in predominant strains of *E. coli* was used as a marker for multiple resistance and compared between groups (23). In analyzing data, we did not adjust for making multiple comparisons (24) since adjusting remains controversial (25,26), particularly for actual observations on nature (27). The association between isolation of resistant strains and specific farming activities and the size of farms was assessed by chi-square analysis.

Results

We matched 113 exposed pig farmers with 113 nonexposed nonfarmers. The overall male-to-female ratio was 6.1, and mean age was 37.8 years (range 21–72). Mean previous time in the professional position occupied at the time of the study was 9.7 ± 1.9 and 13.0 ± 1.6 years for pig farmers and nonfarmers, respectively ($p < 0.01$).

Health insurance reimbursement data showed that antimicrobial agents had been prescribed in the month preceding the study for two pig farmers (one with macrolide and one with broad-spectrum penicillin 24 and 28 days before participation, respectively) and three nonfarmers (one with oral cephalosporin, one with penicillinase-resistant penicillin, and one with tetracycline 3, 10, and 24 days before participation, respectively). However, because of the retrospective nature of this analysis, the low number of participants, the nearly even distribution between pig farmers and nonfarmers, and the fact that reimbursement data are not a formal proof that antimicrobial agents were actually taken, these five persons were included in further analysis. Neither overall, nor class-specific antimicrobial prescriptions during the 6 months preceding participation in the study were significantly different between pig farmers and nonfarmers (Table 1). Prevalence of nasal or pharyngeal isolation of *S. aureus* was significantly higher in pig farmers (PR 1.85; confidence intervals [CI] 1.26 to 2.71]; $p < 0.01$) (Table 2). Isolation of erythromycin-resistant strains was significantly more frequent among *S. aureus* pig farmer carriers than among nonfarmer carriers (PR 9.72; CI 2.53 to 37.30; $p < 0.01$). Moreover, 31 (87%) of 36 macrolide-resistant *S. aureus* isolates from pig farmers were cross-resistant to lincosamides. Five pig farmers, but no nonfarmers, had MRSA (not significant). Analysis of the antimicrobial-susceptibility profile of these strains showed that two were resistant to at least one macrolide antimicrobial agent, four were resistant to aminoglycosides, and four were resistant to pefloxacin. Three of the MRSA carriers had been hospitalized within the 2 years preceding the study, including one within the previous year. The two other farmers had not been hospitalized but had visited outpatient clinics for medical problems within the year preceding the study.

Prevalence of pharyngeal isolation of *Streptococcus pneumoniae*, *H. influenzae*, and β-hemolytic streptococci was low and did not differ significantly between groups (Table 3). One pig farmer carried yeast (*Candida albicans*). Isolation of nongroupable streptococci was frequent and not significantly different between groups, but that of nongroupable streptococci resistant to ampicillin was significantly more frequent in pig farmers than in nonfarmers (PR 2.02; CI 1.32 to 3.09; $p < 0.01$). Prevalence of fecal enterococci was not significantly different between groups nor was isolation of enterococci resistant to erythromycin or vancomycin (Table 4). In all, 16 VRE were isolated including 2 VanA-type *Enterococcus faecium*, along with 11 *E. gallinarum* and 3 *E. casseliflavus* of VanC phenotype and genotype. Nearly all participants carried enterobacteria: 103 (94.5%) of 109 pig farmers and 100 (91.7%) of 109 nonfarmers (PR 1.03; CI 0.96 to 1.10; not significant). Isolation of enterobacteria resistant to nalidixic acid (PR 7.12; CI 2.20 to 23.0; $p < 0.01$), chloramphenicol (PR 2.08; CI 1.17 to 3.68); $p < 0.01$), tetracycline (PR 1.65; CI 1.27 to 2.13; $p < 0.01$), and streptomycin (PR 1.40; CI 1.01 to 1.95; $p < 0.01$) was significantly more frequent in pig

Table 1. Total defined daily doses (DDD) of various classes of antimicrobial agents during the 6 months preceding participation in study[a]

Antimicrobial agent	Total DDD (no. participants[b])			
	Pig farmers		Nonfarmers	
Penicillins (narrow-spectrum, broad-spectrum, and penicillinase-resistant)	138	(9)	132	(9)
Cephalosporins	53	(7)	83	(9)
Macrolides and lincosamides	67	(9)	35	(6)
Others	15	(3)	67	(2)
Total	273	(25)[c]	317	(19)[c]

[a]As determined by health insurance reimbursements to pig farmers and nonfarmers.
[b]Who used any given type of antimicrobial agent.
[c]Some persons had multiple treatments.

Table 2. Nasopharyngeal isolation of *Staphylococcus aureus* with various susceptibility to antimicrobial agents in pig farmers and nonfarmers[a]

Type of *S. aureus*	Prevalence no. (%)				Prevalence ratio	95% CI	p value
	Pig farmers		Nonfarmers				
Any	50/112	(44.6)	27/122	(24.1)	1.85	1.26 to 2.71	<0.01
Resistant to							
Methicillin	5[a]/50	(10.0)	0/27			NA[c]	0.59
Macrolides	36/50	(72.0)	2/27	(7.4)	9.72	2.53 to 37.30	<0.01
Gentamicin	10/50	(20.0)	0/27		NA	NA	0.11
Pefloxacin	8/50	(16.0)	1/27	(3.7)	4.32	0.57 to 32.75	0.22

[a]Matched nasal samples were available for 112 pig farmer–nonfarmer pairs only.
[b]In addition to being resistant to methicillin, two strains were resistant to at least one macrolide antibiotic (two were resistant [R] to erythromycin, lincomycin, and pristinamycin; 1 susceptible [S] to erythromycin only; and one susceptible to pristinamycin only), 4 strains were R to aminoglycosides (2 were RRS and 2 RRR to kanamycin, tobramycin, and gentamicin, respectively). Four strains were resistant to pefloxacin.
[c]NA, not applicable.

farmer carriers of enterobacteria than in nonfarmer carriers. Regarding the predominant flora, the most frequent species isolated were *Escherichia coli* (917/995; 92.2%) followed by *Hafnia alvei* (48/995; 4.8%) and *Citrobacter freundii* (11/995; 1.1%) with no significant between-group differences. The prevalence of isolation of *E. coli* resistant to cotrimoxazole (PR 3.02; CI 1.68 to 5.44; $p < 0.01$), tetracycline (PR 2.22; CI 1.48 to 3.32; $p < 0.01$), streptomycin (PR 1.40; CI 1.01 to 1.95; $p = 0.04$), or nalidixic acid (PR not calculable; $p < 0.01$) was significantly higher in pig farmers carrying *E. coli* than in nonfarmers (Table 4). In all instances in which subgroups of pig farmers and nonfarmers were compared, no significant between-group difference emerged in terms of age, sex, and county of residence. Prevalence of co-resistance to ampicillin, streptomycin, and cotrimoxazole was also significantly higher in *E. coli* from pig farmers (24%, 24/100) than from nonfarmers (12.2%, 12/98) (PR 1.96; CI 1.04 to 3.70; $p = 0.03$). No strains resistant to ceftazidime were isolated. No strains of *Clostridium difficile*, *Pseudomonas aeruginosa*, or *Staphylococcus aureus* were isolated from the feces of any study participant. Prevalence of yeast was not significantly different between pig farmers and nonfarmers, and the species were evenly distributed (Table 4).

Most pig farmers had several professional activities. Only a few farmers used isolation precautions (Table 5). We found no statistical association between professional activity or use of masks and gloves and the prevalence of resistant bacteria. By contrast, prevalence of nasal isolation of *S. aureus* resistant to macrolides increased significantly, from 33% (5/15) in pig farmers working in farms raising 84–180 swine, to 70% (7/10), 92% (11/12), and 100% (13/13) in those working in farms raising 181–270, 271–399, and >400 swine, respectively (chi-square linear slope; $p < 0.01$).

Discussion

Our results showed that the prevalence of antimicrobial drug resistance in bacteria from the nasal, pharyngeal, and fecal flora was higher in pig farmers than in nonfarmers. With a few exceptions, pig farmers and nonfarmers had not taken antimicrobial agents during the month preceding the study and had not been differentially exposed to such agents during the previous 6 months. That *E. coli* (11–13)

Table 3. Pharyngeal isolation of selected microorganisms in pig farmers and nonfarmers[a,b]

Microorganisms	Prevalence, no. (%)				Prevalence ratio	95% CI	p value
	Pig farmers		Nonfarmers				
Streptococcus pneumoniae	0/112	(0)	3/112	(2.7)	NA	NA	0.25
Haemophilus influenzae	6/112	(5.4)	5/112	(4.5)	1.20	0.38 to 3.82	1.00
Enterobacteria	1/112	(0.9)	2/112	(1.8)	0.50	0.05 to 5.44	1.00
Yeasts[c]	1/112	(0.9)	0/112		NA	NA	0.25
β-hemolytic streptococci[d]	11[e]/112	(9.8)	9[f]/112	(8.0)	1.22	0.53 to 2.83	0.82
NGS[g]							
Any	108/112	(96.4)	100/112	(89.3)	1.08	1.00 to 1.16	0.06
Resistant to							
Ampicillin	48/108	(44.4)	22/100	(22.0)	2.02	1.32 to 3.09	<0.01
Macrolides	108/108	(100.0)	100/100	(100.0)	NA	NA	1.00

[a]Matched pharyngeal samples were available for 112 pig farmer–nonfarmer pairs.
[b]CI, confidence interval; NA, not applicable.
[c]*Candida albicans*.
[d]Several species were present in some study participants.
[e]Group A streptococcus:1, group C: 5, *S. anginosus*: 3, *S. intermedius*: 1, *S. constellatus*: 4.
[f]Group A streptococcus: 1, group C: 5, *S. anginosus*: 3, *S. intermedius*: 1, *S. constellatus*: 3.
[g]Nongroupable streptococci.

Table 4. Fecal isolation of selected microorganisms in pig farmers and in nonfarmers

Microorganisms	Prevalence no. (%)				Prevalence ratio	95 % CI	p value
	Pig farmers		Nonfarmers				
Enterococci							
Any	71/109	(65.1)	80/109	(73.4)	0.89	0.75 to 1.05	0.21
Resistant to							
Erythromycin	38/71	(53.5)	46/80	(57.5)	0.93	0.70 to .24	0.62
Vancomycin	6[b]/71	(8.5)	10[c]/80	(12.5)	0.68	0.26 to 1.77	0.42
Enterobacteria[d]							
Any	103/109	(94.5)	100/109	(91.7)	1.03	0.96 to 1.10	0.58
Resistant to							
Ampicillin	68/103	(66.0)	55/100	(55.0)	1.20	0.96 to 1.50	0.11
Ceftazidime	0		0		NA[e]	NA	NA
Streptomycin	69/103	(67.0)	48/100	(48.0)	1.40	1.09 to 1.78	<0.01
Kanamycin	29/103	(28.2)	23/100	(23.0)	1.22	0.76 to 1.96	0.40
Gentamicin	10/103	(9.7)	3/100	(3.0)	3.24	0.92 to 11.42	0.05
Chloramphenicol	30/103	(29.1)	14/100	(14.0)	2.08	1.17 to 3.68	<0.01
Tetracycline	73/103	(70.9)	43/100	(43.0)	1.65	1.27 to 2.13	<0.01
Nalidixic acid	22/103	(21.4)	3/100	(3.0)	7.12	2.20 to 23.0	<0.01
Escherichia coli[f]							
Any	100/109	(91.7)	98/109	(89.9)	1.02	0.94 to 1.10	0.64
Resistant to							
Ampicillin	36/100	(36.0)	34/98	(34.7)	1.04	0.71 to 1.51	0.85
Ceftazidime	0		0		NA	NA	NA
Streptomycin	50/100	(50.0)	35/98	(35.7)	1.40	1.01 to 1.95	0.04
Kanamycin	10/100	(10.0)	12/98	(12.2)	0.82	0.37 to 1.80	0.62
Gentamicin	2/100	(2.0)	0		NA	NA	0.99
Chloramphenicol	11/100	(11.0)	9/98	(9.2)	1.20	0.52 to 2.76	0.67
Tetracycline	52/100	(52.0)	23/98	(23.5)	2.22	1.48 to 3.32	<0.01
Cotrimoxazole	37/100	(37.0)	12/98	(12.2)	3.02	1.68 to 5.44	<0.01
Nalidixic acid	11/100	(11.0)	0		NA	NA	<0.01
Staphylococcus aureus	4/109	(3.7)	2/109	(1.8)	2.0	0.37 to 10.69	0.68
Yeasts	19[g]/109	(17.4)	18[h]/109	(17.4)	1.06	0.59 to 1.90	1.00

[a]Matched fecal samples were available for 109 pig farmers and nonfarmer pairs only.
[b]*Enterococcus faecium*: 0, *E. gallinarum*: 6.
[c]*E. faecium*: 2, *E. gallinarum*: 5, *E. casseliflavus*: 3.
[d]Using direct plating plating on Drigalski agar without or with antimicrobial agents (first technique, see Methods).
[e]NA, not applicable.
[f]From the predominant fecal flora (second technique, see Methods).
[g]*Candida albicans*: 1, *Geotrichum* sp.: 15, *C. glabrata*: 2, *Rhodolulora* sp.: 1.
[h]*C. albicans*: 2, *Geotrichum* sp.: 14, *Saccharomyces cerevesia*: 2.

and enterococci (14) are significantly more resistant in persons working in farms or slaughterhouses than in urban residents had been reported, but a potential role of antimicrobial treatments in these workers could not be excluded and the increased prevalence of carriage of resistant organisms had not been quantified.

The prevalence of *S. aureus* nasal carriage in nonfarmers was similar to that reported previously in the general population (28), which suggests that the higher isolation rate in pig farmers was due to their work environment. This hypothesis was further supported by the increased resistance to macrolides (still the fourth most common class of antimicrobial agents used in food production [8]) of *S. aureus* isolates from pig farmers and the link between this resistance and the size of the farm. Why the isolation rate of *S. aureus* was higher in pig farmers remains unclear. Several hypotheses, including high transfer of animal specific clones, should be raised and investigated.

In the pharynx, ampicillin resistance of nongroupable streptococci in pig farmers may contribute to further transfer of β-lactam resistance to *Streptococcus pneumoniae* by transformation (29). In the feces, antimicrobial drug resistance in enterobacteria was also greater in pig farmers for four of eight markers tested in the subdominant flora, and for four of nine markers in the predominant flora. Resistance in *E. coli* was close to that of healthy participants from developing countries (22). The prevalence of resistance in enterobacteria from the subdominant flora of our nonfarmers was lower than that in participants of the only study published that used the same methods; however, that study included mostly laboratory workers (A. Andremont, pers. comm.), who are known to be more colonized by resistant enterobacteria than are urban and rural dwellers (30). The rate of VRE colonization that we observed differed from that reported in France (31), which might be due to the enrichment step we used; however, the

Table 5. Frequency of use of masks and gloves by 113 pig farmers during selected farming activity

Activity	No. (%) with that activity	No. (%) using[a]	
		Masks	Gloves
Food preparation, daily or often	109 (96)	4 (3.6)	8 (7.3)
Manual food distribution, handling, or mixing	78 (69)	4 (5.2)	5 (6.4)
Handling of pig feces, daily or often	87 (77)	2 (2.3)	7 (8.0)
Antibiotic administration to animals	112 (99)	4 (3.5)	9 (8.0)

[a]During that activity.

rate of VRE colonization did not differ between farmers and nonfarmers. This finding suggests that the 1997 ban (32) of avoparcin, a glycopeptide previously used as a growth promoter, was effective. Although specific information on avoparcin is lacking, 145 tons of antimicrobial agents were used globally in France in 1998 in pig raising, including 70 mg of growth additive per kilogram of pork meat produced (33).

Three possible explanations may explain why isolation of resistant bacteria in pig farmers was higher than in nonfarmers. First, farmers may come in contact with more antimicrobial-resistant bacteria from pigs; these bacteria are then transferred to the farmers. Second, farmers may be in frequent contact with antimicrobial agents themselves or antimicrobial residues that are given to the pigs in the workplace. The third possibility is that farmers receive more antimicrobial agents for other, i.e., medical, reasons. The first of these possibilities appears most likely because 1) farmers used very few precautions during contact with animal feces, 2) antimicrobial exposure is a well-known risk factor for intestinal yeast colonization (34,35), and yeast colonization in both groups was low, and 3) antimicrobial prescriptions were not significantly different between pig farmers and nonfarmers during the previous 6 months.

We did not assess the use of antimicrobial agents for animals in each of the 113 farms where pig farmers worked. However, 1,364 tons of antimicrobial agents were sold in France in 1999 for veterinary medicinal use. Of these, tetracycline, cotrimoxazole, and β-lactams together accounted for 79.5% (8), a finding compatible with the high resistance rates found in pig farmers. However, we could not assess the exact cause of the high antimicrobial resistance rates in farmers. Determining the exact cause may not be as important as the fact that these people are colonized with a much higher rate of resistant bacteria. Further studies will need to be undertaken to identify the cause of this phenomenon.

Food products are a source of resistant bacteria (9,10). We minimized the risk that differences in food intake caused the higher prevalence of resistance in pig farmers by matching pig farmers with nonfarmers by age, sex, and county of residence. Children can be a source of resistant bacteria in households (36) and thus might be a confounding factor if the number of children was greater in pig farmer families than in nonfarmer families. However, this factor was not documented in the study questionnaire and thus could not be investigated.

Some inherent limitations of cross-sectional studies invite cautious assessments of our results. The lack of preexposure data on resistance and the general design of the study preclude determining a causal relationship between exposure and acquired resistance. However, the observation we made indicates that professional pig farming is significantly associated with isolation of antimicrobial-resistant commensal species. The minimal use of contact precautions by pig farmers may have further increased this risk, but the study was not designed to assess the efficacy of contact precautions, and thus no recommendations can be drawn in this matter.

Pigs could be raised with considerably fewer antimicrobial agents than currently used, and many animals can be raised with little or no exposure to such drugs at all (37). However, antimicrobial agents will still be used to treat sick animals. Additional studies are needed to evaluate the consequences of isolating resistant bacteria in farmers and, if necessary, design appropriate preventive measures.

Acknowledgments

We thank J. Bordet, R. Camus, R. Carozzani, M.F. Darchy, N. Fily, P. Gales, J. Gaudon, M. Harrewyn, C. Le Henaff, Y. Koskas, E. Lecocq, A. Lozach, J.L. Mary, P.Morriseau, N. N'Guyen, J.C. Presle, D. Peron, J. Ribbe, M. Roy, J. Roze, G. Savatier, who recruited the study participants, interviewed them, and obtained the primary samples; M. Goldberg, H. de Valk, M. Valenciano, and D. Daube for discussion; V. Jarlier and l'Observatoire de l'Epidémiologie de la Résistance aux Antibiotiques for providing a questionnaire during the investigation of contacts from methicillin-resistant *Staphylococcus aureus* carriers; and G.B. Pier for critical reading of the manuscript.

This work was supported in part by contract AC003E from the Ministère de l'Aménagement du Territoire et de l'Environnement (Programme de Recherche Environnement et Santé 1999) and by a grant from Mutualité Sociale Agricole, France. This work was presented in part at the 32nd ICAAC September 2002, San Diego, California.

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Endemic Venezuelan Equine Encephalitis in Northern Peru

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Since Venezuelan equine encephalitis virus (VEEV) was isolated in Peru in 1942, >70 isolates have been obtained from mosquitoes, humans, and sylvatic mammals primarily in the Amazon region. To investigate genetic relationships among the Peru VEEV isolates and between the Peru isolates and other VEEV strains, a fragment of the PE2 gene was amplified and analyzed by single-stranded conformation polymorphism. Representatives of seven genotypes underwent sequencing and phylogenetic analysis. The results identified four VEE complex lineages that cocirculate in the Amazon region: subtypes ID (Panama and Colombia/Venezuela genotypes), IIIC, and a new, proposed subtype IIID, which was isolated from a febrile human, mosquitoes, and spiny rats. Both ID lineages and the IIID subtype are associated with febrile human illness. Most of the subtype ID isolates belonged to the Panama genotype, but the Colombia/Venezuela genotype, which is phylogenetically related to epizootic strains, also continues to circulate in the Amazon basin.

Venezuelan equine encephalitis virus (VEEV) is an emerging mosquito-borne RNA virus in the family *Togaviridae*, genus *Alphavirus*. Since its isolation in 1938, many equine epizootics and epidemics have been reported in Colombia, Venezuela, Trinidad, Peru, Ecuador, Mexico, and the United States, and other countries (1–5); the number of reports of human and equine cases have increased during recent years (6–8). At least 13 distinct subtypes and varieties, including several different species, make up the VEE complex (Table 1). Only subtype I varieties A, B, and C have caused major outbreaks involving hundreds of thousands of equine and human cases. Subtypes II through VI and subtype I varieties D, E, and F are enzootic, equine-avirulent strains not associated with major equine outbreaks or epidemics, although they do cause human illness, which can be fatal (9,10).

In Peru, VEEV was first isolated in the 1940s, when subtype IAB strains caused equine epizootics and epidemics along the Pacific coast (7,8). Field investigations were later conducted to determine the origin of the IAB strains. Initial ecologic studies in the Amazon region of northeastern Peru (11,12) yielded 11 VEE complex isolates from mosquitoes and sentinel hamsters during 1970 and 1971 in Quistococha, near Iquitos (Figure 1). Antigenic analyses identified 10 isolates as subtype ID VEEV (11) and one strain as subtype IIIC in the VEE complex (12,13). Because these viruses were isolated only from sentinel hamsters and mosquitoes, whether they were human pathogens remained unclear. Not until 1993 through 1995, when VEEV subtype ID was isolated from persons with febrile illness in the Amazon region of Peru (4,14), was there clear evidence that VEEV causes human disease in this region. Two subtype ID strains were isolated from patients in Pantoja in 1994, and 10 other subtype ID strains were obtained from febrile patients in and around the city of Iquitos from 1993 to 1995.

Nucleotide sequences and phylogenetic analyses recently performed on Peruvian VEEV isolates indicated that the 1970s VEEV subtype ID mosquito isolates and the 1994 Pantoja human isolates belong to the Colombia/Venezuela ID genotype, whereas the 1993–1995 Iquitos

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Table 1. Classification of the Venezuelan equine encephalitis (VEEV) complex of alphaviruses

Subtype	Variety	Species	Transmission pattern	Equine virulence[b]	Location	Vector
I	AB	VEEV	Epizootic	Virulent	Central, South, North America	Mammalophilic mosquitoes
	C	VEEV	Epizootic	Virulent	South America	Mammalophilic mosquitoes
	D	VEEV	Enzootic	Avirulent	Central, South America	*Culex (Mel.) ocossa, panocossa, pedroi, adamesi, vomerifer*
	E	VEEV	Enzootic	Variable	Central America, Mexico	*Cx. (Mel.) taeniopus*
	F	Mosso das Pedras	Enzootic	Unknown	Brazil	Unknown
II		Everglades	Enzootic	Avirulent	Southern Florida	*Cx (Mel.) cedecei*
III	A	Mucambo	Enzootic	Avirulent	South America	*Cx (Mel.) portesi*
	B	Tonate	Enzootic	Unknown	South, North America	Unknown (Tonate), bug (Bijou Bridge strains from N. America)
	C		Enzootic	Unknown	Peru	Unknown
IV		Pixuna	Enzootic	Unknown	Brazil	Unknown
V		Cabassou V	Enzootic	Unknown	French Guiana	Unknown
VI		Rio Negro	Enzootic	Unknown	Argentina	Unknown

[a]Source: The Arboviruses: Epidemiology and Ecology (9).
[b]Virulent strains produce high viremia titers and generally ≥50% death rates in horses experimentally infected.

human isolates belonged to the Panama ID genotype (9). Lack of detection of the Colombia/Venezuela genotype in the Iquitos area during 1993 through 1995, when the Panama genotype was repeatedly isolated, suggested that the Colombia/Venezuela genotype had been replaced by virus strains from Panama. Because the Colombia/Venezuela VEEV genotype is believed to give rise to epizootic viruses through mutations of the E2 envelope glycoprotein (15–18), disappearance of this genotype from the Iquitos area could have important public health implications.

Since 1995, 75 VEEV strains have been isolated from mosquitoes, humans, and sentinel hamsters in Peru. We determined the genetic relationships among strains isolated from 1995 through 2002 and compared them with other VEEV strains isolated from the Americas. Our results indicate that the VEEV subtype ID Colombia/Venezuela genotype continues to circulate in Iquitos but may infect people at a lower rate than the Panama genotype. We also demonstrated that a recent subtype III isolate is genetically distinct from the subtype IIIC strain isolated in 1971 (12). This strain, which we propose as subtype IIID in the VEE complex, and both subtype ID genotypes cause human febrile disease in the Iquitos area.

Methods

Study Sites

VEEV isolates were obtained from several locations in Peru. The main study site was centered around Iquitos, a city of 300,000 people on the Amazon River in the Department of Loreto (Figure 1). The major occupations of the inhabitants are housekeeping, teaching, military work, agriculture, fishing, and tourism. The climate is tropical, with a mean annual temperature of 27.5°C and mean annual precipitation of 2.7 m (4).

Virus Isolates

The VEE complex isolates included in this study are shown in Table 2. These viruses were provided by the University of Texas Medical Branch World Health Organization Collaborating Reference Center for Arboviruses, the United States Army Medical Research Institute of Infectious Diseases, and the U.S. Naval

Figure 1. Map of Peru showing the geographic distribution of the Venezuelan equine encephalitis virus (VEEV) complex isolates included in the study. Numbers in parenthesis indicate the number of isolates compared to the total number of febrile cases during the year. ID, IAB, IIIC, IIID correspond to VEEV subtypes isolated during the indicated year.

Table 2. Venezuelan equine encephalitis complex virus isolates included in this study

Subtype	Code	Location	Year	Host	Signs and symptoms[a]
ID	IQT0988	Sanidad EP/Vargas Guerra, Iquitos	1993	Human	F, A
ID	IQT1015	Sanidad EP/Vargas Guerra, Iquitos	1993	Human	F, H, C
ID	IQT1026	Sanidad EP/Vargas Guerra, Iquitos	1994	Human	F, H, E, B, D, C, G, N, S
ID	DEI5191	Pantoja, Iquitos	1994	Human	F, C, H, B
ID	DEI5193	Pantoja, Iquitos	1994	Human	F, C, I, H, B, N, V
ID	IQT1042	Dir. Reg Salud de Loreto, Iquitos	1994	Human	F, H, B, V
ID	IQT1071	Pedrelor, Iquitos	1994	Human	F, H, B, E, A, I, C
ID	IQT1081	Pedrelor, Iquitos	1994	Human	F, H, B, E, D, R, C
ID	IQT1085	Hospital Regional Amazonas, Iquitos	1994	Human	F, H, B, D, G, N, S
ID	IQT1098	Sanidad EP/Vargas Guerra, Iquitos	1994	Human	F, H, E, B, D, C, S
ID	IQT1101	Pedrelor, Iquitos	1994	Human	F, H, E, B, D, V, C, G
ID	IQT1120	Sanidad EP/Vargas Guerra, Iquitos	1994	Human	F, H, B, D, V, C
ID	IQT1724	Pedrelor, Iquitos	1995	Human	F, H, E, B, D, V, C, I, G
ID	IQT1735	Pedrelor, Iquitos	1995	Human	F, H, B, E, D, R, V, C
ID	IQT3745	Sanidad EP/Vargas Guerra, Iquitos	1997	Human	F, H, E, B, D, G
ID	IQT3971	San Antonio, Iquitos	1997	Human	F, H, E, B, D, V, I, C
ID	IQT4091	Bellavista, Iquitos	1997	Human	F, B, D, V, I, M, V, S, J
ID	IQT4177	Sanidad EP/Vargas Guerra, Iquitos	1997	Human	F, H, E, B, D, V, C, G
ID	IQT4191	Bellavista, Iquitos	1997	Human	F, H, E, B, D, C, S
ID	PC28	Iquitos	1997	Rodent (*Proechimys* spp.)	
III	PC254	Iquitos	1997	Rodent (*Proechimys* spp.)	
III	PC256	Iquitos	1997	Rodent (*Proechimys* spp.)	
ID	IQT5798	San Juan, Iquitos	1998	Human	F, H, E, B, D, C
ID	IQT5831	Cardoso, Iquitos	1998	Human	F, H, E, B, D, C
ID	IQT5876	San Juan, Iquitos	1998	Human	F, H, E, B, D, V, C
ID	IQT5885	Moronacocha Iquitos	1998	Human	F, H, E, B, D, V, G, T
ID	IQT6088	9 de Octubre, Iquitos	1998	Human	F, H, E, B, D, V, I, C, G
ID	IQT6119	San Juan, Iquitos	1998	Human	F, H, E, B, D, R, V, I, C
ID	IQT6415	San Juan, Iquitos	1998	Human	F, H, E, B, D, V, I, C
ID	IQT6486	9 de Octubre, Iquitos	1998	Human	F, H, E, B, D, V
ID	IQT6674	Tupac Amaru, Iquitos	1998	Human	F, H, E, B, D, C
ID	IQT6712	Hospital Apoyo, Iquitos	1998	Human	F, H, E, B, D, V, S
ID	IQT6937	NMRCD, Iquitos	1998	Human	F, H, E, B, D, V, S
ID	IQT7057	San Juan, Iquitos	1998	Human	F, H, E, D, V, C
ID	IQT7060	San Juan, Iquitos	1998	Human	F, H, E, B, D, C
ID	IQT7327	Tupac Amaru, Iquitos	1998	Human	F, H, E, B, D, V, C, G
ID	IQT7460	CIA Petrolera, Iquitos	1998	Human	F, H, B, C
ID	IQT7988	Belen, Iquitos	1998	Human	F, H, B, D, V, C, G, S
ID	IQT8131	Belen, Iquitos	1998	Human	F, H, E, B, D, R, V, C, D
ID	IQT8558	San Juan, Iquitos	1998	Human	F, H, B, D, V, I, C, G
ID	PE30609	Iquitos	1998	Mosquito	
III	PE407660	Iquitos	1998	Mosquito	
III	PE409040	Iquitos	1998	Mosquito	
III	PE409100	Iquitos	1998	Mosquito	
ID	IQU0465	San Antonio, Iquitos	1999	Human	F, H, E, B, D, R, C
ID	IQU0664	San Antonio, Iquitos	1999	Human	F, H, E, B, D, C
ID	IQU0890	6 de octubre, Iquitos	1999	Human	F, H, B, E, D, V, C
ID	IQU0953	6 de octubre, Iquitos	1999	Human	F, H, B, E, C
ID	IQU1050	Belen, Iquitos	1999	Human	F, H, B, E, V, C
ID	IQU1106	San Antonio, Iquitos	1999	Human	F, H, E, B, A, V, C
ID	IQU1217	Belen, Iquitos	1999	Human	F, H, E, B, D, V, I, C, G
ID	IQU1279	Belen, Iquitos	1999	Human	F, H, E, B, V, C, G
ID	IQU1282	Cardozo, Iquitos	1999	Human	F, H, E, B, D, C
ID	IQU1318	Tupac Amaru, Iquitos	1999	Human	F, H, E, B, D, V, C
ID	IQU1341	Zungarococha, Iquitos	1999	Human	F, H, E, B, D, R, V, C
ID	IQU1402	Cardozo, Iquitos	1999	Human	F, H, E, B, A, V, C, G, S
ID	IQU1718	San Antonio, Iquitos	1999	Human	F, H, E, B, D, I, C
III	FSL0190	San Juan, Iquitos	2000	Human	F, C, L
ID	FSL0201	San Juan, Iquitos	2000	Human	F, C, L, D, B, A
ID	FSL0205	San Juan, Iquitos	2000	Human	F, C, L, D, B, A
ID	FSL0240	San Juan, Iquitos	2000	Human	F, C, L, D, A, I, V, J, G
ID	FSL0252	San Juan, Iquitos	2000	Human	F, C, L, D, B, A
ID	IQU3026	Hospital Apoyo, Iquitos	2000	Human	F, H, E, B, D, V, C, S
ID	FSL0507	Hospital Militar, Iquitos	2001	Human	F, H
IIIC	54-001	Iquitos	2002	Hamster	

[a]A, abdominal pain; H, headache; F, fever; C, chills; E, eye pain; B, body pain; D, arthralgia; G, cough; N, nasal congestion; S, sore throat; I, diarrhea; V, vomits; R, rash; M, melena; J, jaundice; T, hematuria; L, malaise.

Medical Research Center Detachment, Lima, Peru (NMRCD). Most of the viruses were isolated in Vero cells from serum samples of febrile patients, sentinel hamsters, and mosquitoes. These viruses were passaged once in Vero cells, and supernatant fluid was stored at –70°C for subsequent extraction of viral RNA.

Extraction of Viral RNA and cDNA Synthesis

Viral RNA was extracted from cell culture media as described elsewhere (19). Briefly, 250 μL of infected cell culture supernatant was mixed with 750 μL of Trizol LS (Gibco BRL, Bethesda, MD), and RNA was extracted following the manufacturer's protocol. For cDNA synthesis, 5 μL of the extracted RNA was mixed with 1 μmol of reverse primer V9207B (Table 3), 1x First Strand Buffer (Gibco BRL, Bethesda, MD), 1 mmol deoxynocleoside triphosphate (dNTPs), 80U RNAsin (Promega, Madison, WI), and 200 U SuperScript II Reverse Transcriptase (Gibco BRL, Bethesda, MD), and then incubated at 42°C for 1 h.

PCR Amplification

Primers used for the polymerase chain reaction (PCR) and sequencing are listed in Table 3. For amplification of the N-terminus of the PE2 envelope glycoprotein precursor gene, primers V8369(+) and V9207B(–) were used, and the resulting product was sequenced with primers V8659(+) and V8953(–) (19). PCRs included 2.5 U of Taq polymerase (Promega, Madison, WI), 1X Promega Taq buffer, 300 nmol of each primer, 1 mmol $MgCl_2$, 0.2 mmol dNTPs, and 10 μL of the cDNA reaction; 30 amplification cycles included heat-denaturation at 95°C for 30 s, primer annealing at 48°C for 30 s, and extension at 72°C for 1 min. A final extension of 10 min was used to ensure complete double-stranded DNA synthesis. In the case of the proposed subtype IIID strains, the reverse primer used for cDNA synthesis and PCR was V9257B(–). In some cases, PE2 amplicons were not obtained, and those isolates were amplified by using alphavirus-specific nsP1 primers described previously (20). The complete 26S gene of one subtype IIID strain (human isolate FSL190) was amplified and sequenced for further comparison with other VEE complex subtypes; two overlapping PCR amplicons were obtained by using primer pairs E/V 7514(+)/VIIID 10471 (–), and α10247A(+)/Mlu-T25(–), and sequenced by gene walking.

Single-Stranded Conformation Polymorphism (SSCP)

SSCP analysis was performed as previously described (16). PCR products were purified with the Qiaquick gel extraction kit (Qiagen, Valencia, CA), and 2 μL of the purified PCR amplicon DNA was mixed with 8 μL of SSCP loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol). To denature the DNA, the reaction was heated to 95°C for 5 min and immediately cooled on ice, loaded onto an 8% polyacrylamide gel, and electrophoresed in 1 x Tris-borate-EDTA buffer at room temperature for 20 h at 8 mA. The single-stranded DNA products were visualized with silver staining (21). Single-strand conformational polymorphism genotypes were determined by comparing the migration patterns of the double-stranded DNA of the various isolates with one another and against a standard DNA ladder. Unique migration patterns were designated as distinct SSCP genotypes.

Table 3. Oligonucleotides used for polymerase chain reaction amplification and sequencing analysis

Primer (genetic sense)	Sequence (5′→3′)
V8369(+)	GAGAACTGCGAGCAATGGTCA
V9207B(–)	TRCACTGGCTGAACTGTT
V9257B(–)	TACACCCAYTTRTCRTTCTG
V8659(+)	AATTGAGGCAGTGAAGAGCGAC
V8953(–)	CTGCCTACAGGATTAAAT
E/V 7514(+)	ACYCTCTACGGCTRACCTRA
VIIID 10471(–)	CCTTCCGGTCGAACGGGGTCC
α10247A(+)	TACCCNTTYATGTGGG
Mlu-T25 (–)	TTACGAATTCACGCGTTTTTTTTTTTTTT TTTTTTTTTT

Sequencing and Phylogenetic Analysis

Two or three representatives of each SSCP genotype were selected randomly for sequencing and phylogenetic analysis. Most PCR products were sequenced directly with an Applied Biosystems (Foster City, CA) Prism automated DNA sequencing kit according to the manufacturer's protocol. In some cases, PCR products were cloned into the PCR II vector (Invitrogen, Carlsbad, CA), and at least two clones were sequenced by using vector-specific primers. Sequences were aligned by using the Clustal program in the MacVector (Oxford Molecular Group, Campbell, CA) software package, and phylogenetic analyses were conducted by using maximum parsimony, neighbor-joining, and maximum likelihood programs implemented in the PAUP 4.0 software (22). For the neighbor joining analysis, the HKY85 distance formula was used. Bootstrap analyses were conducted with 1,000 replicates to place confidence values on groupings within trees (23).

Plaque Reduction Neutralization Tests

To obtain immune sera against VEEV subtypes IIIA, IIIB, IIIC and IIID, cotton rats (*Sigmodon hispidus*, Harlan, Indianapolis, IN) were infected subcutaneously with 1,000 PFUs of virus. Three weeks after injection, the animals were bled for antibody testing using plaque reduction neutralization tests (PRNT) with Vero cells (24). Viruses were tested against all antibody preparations, and the endpoint titers were defined as the reciprocal of the

highest dilution inhibiting ≥80% of approximately 100 PFU of virus.

Results

Distribution and Characterization of Human VEEV Cases in Iquitos

Studies to identify the cause of human febrile illness in the Amazon region of Peru since 1995 (Watts et al., unpub. data) identified at least 183 cases of VEEV on the basis of immunoglobulin (Ig) M antibody detection, virus isolation, or both. The VEEV cases in the Iquitos area were localized into two major clusters: the region surrounding Lake Morona Cocha, which typically floods during the rainy season, and cases near the Itaya and the Amazon rivers (Figure 2). Cases were also observed to the north and southwest of Iquitos. Most patients with VEEV did not report travel outside their home region, suggesting that many infections occurred in the city of Iquitos. Common signs and symptoms of VEEV infection included fever, headache, chills, malaise, diarrhea, vomiting, arthralgia, and abdominal pain. Clinical information obtained by NMRCD provided no indication of neurologic disease, and no deaths were reported.

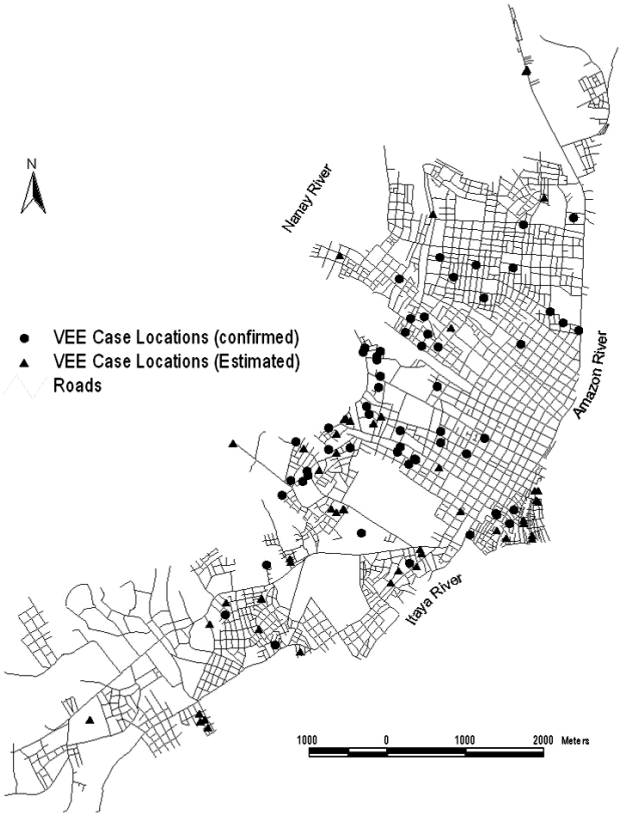

Figure 2. Map of Iquitos showing the locations of human Venezuelan equine encephalitis virus cases in the city.

Sequencing and Phylogenetic Analysis

An 856-bp fragment from the N terminus of the PE2 gene was obtained for most of the VEEV subtype ID isolates using primers V8369(+)/V9207(–). This region was chosen because it has been used previously in similar phylogenetic analyses, resulting in a large database that allows for genetic comparison with new isolates (4,9,25). In addition, the fragment sequenced contains the N-terminal portion of the E2 glycoprotein gene, which has been shown to undergo critical amino acid substitutions associated with epizootic VEEV emergence (18).

To screen the amplified isolates for genetic differences and to eliminate the cost and effort in sequencing each virus isolate, SSCP analyses were performed as previously described (19). At least 2–3 representatives of each SSCP genotype were selected at random for sequence and phylogenetic analysis. Some isolates could not be amplified with primers V8369(+)/V9207B(–), and later phylogenetic analyses indicated that they corresponded mainly to subtypes other than ID. For these isolates, primer V9257(–) was substituted for V9207B(–) in the cDNA syntheses and PCR amplifications. In addition, alphavirus consensus primers (20) that amplify a portion of the nsP1 gene were used to confirm the identity of these isolates and to look for evidence of recombination within the VEE complex (data not shown).

Phylogenetic analyses performed using maximum parsimony, neighbor-joining, and distance-matrix methods generated similar tree topologies. The neighbor-joining tree based on the PE2 gene (Figure 3) showed that the newly sequenced VEEV Peruvian strains grouped into four major clades: the subtype ID Colombia/Venezuela genotype, the ID Panama/Peru genotype, subtype IIIC isolated in 1971 in the Iquitos area of Peru from a mosquito, and a newly identified group of viruses that fell within subtype III but were distinct from IIIC, which we propose as a new genetic subtype IIID.

The greatest number of human isolates from Peru fell into VEEV subtype ID. In agreement with previous studies (9), genetic analysis of the ID strains delineated two distinct genotypes that differ by approximately 5% in their nucleotide sequences: the Colombia/Venezuela genotype, which is believed to have generated the epizootic IAB and IC viruses (15–18), and the Panama/Peru genotype. The Colombia/Venezuela ID genotype was represented by isolates in Peru during the 1970s and two isolates made from Pantoja in 1994. Previous data suggested that this genotype was no longer circulating in Iquitos and had been replaced by the Panama genotype; however, the evidence that these viruses continued to circulate in Iquitos was supported by human isolates from 1998 and 2002, which grouped into the Colombia/Venezuela clade with strong

bootstrap support (Figure 3). However, most of the Peruvian VEEV isolates from the Iquitos region grouped into the ID Panama/Peru genotype and were obtained from humans, rodents, and mosquitoes.

The VEE complex subtype IIIC strain, first isolated in 1971 from mosquitoes collected near Iquitos (12,13), was isolated again in 2002 from a sentinel hamster in the Amazon region. Several other isolates from mosquitoes, humans, and rodents also grouped into subtype III (88% bootstrap support) but were quite distinct from the subtype IIIC isolates (18% nucleotide sequence, 8% amino acid sequence divergence in the PE2 protein genes). These levels of divergence are equal to or greater than those of other VEE complex antigenic subtypes, so we propose the designation of subtype IIID for these strains.

To further document these relationships within subtype III and to investigate possible recombination, phylogenetic trees on the basis of the nsP1 gene were also constructed. The nsP1 trees did not show any evidence of recombination and also supported the sister-group relationship between subtype IIIC and the related IIID isolates (data not shown). To further genetically characterize this new subtype III strains, trees based on the complete 26S structural gene sequences were generated. All trees, including the neighbor-joining tree shown in Figure 4, confirmed the close relationship between the IIIC and IIID subtypes, as well as their level of divergence.

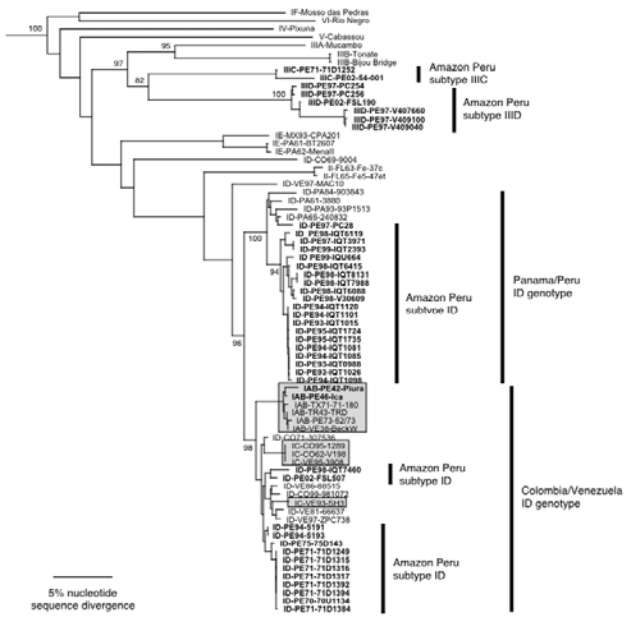

Figure 3. Phylogenetic tree of the Venezuelan equine encephalitis virus (VEEV) complex derived from partial PE2 gene sequences of Peruvian VEEV isolates and homologous sequences published previously, using the neighbor joining program implemented in PAUP 4.0 (22). The tree was rooted using an outgroup comprised of four major lineages of Eastern Equine Encephalitis virus (26). Virus strains are labeled by VEE complex subtype, abbreviated country (FL=Florida, USA) and year of isolation, followed by strain designation. Numbers indicate bootstrap values for clades to the right.

Antigenic Characterization of Proposed Subtype IIID

To characterize antigenically the putative new VEEV subtype IIID, PRNTs were performed to determine the antigenic relationships among the subtype III viruses (Table 4). Mucambo virus (subtype IIIA) versus Tonate virus (subtype IIIB) showed a fourfold difference in only one direction, which, based on the traditional serologic classification criteria (26), indicates that these are virus subtypes. When subtype IIIC was compared with the IIIA and IIIB subtypes, a fourfold or greater difference was observed in both directions, indicating that IIIC is a different virus. However, only a twofold difference existed in endpoint titers between subtype IIIC and the new IIID, which indicated that they are not distinct subtypes according to traditional antigenic criteria (28).

Discussion

VEEV is considered an emerging human pathogen in Latin America because a resurgence of VEE disease has occurred in Mexico and South America during the past decade (8). Our results indicate that VEEV is also endemic in Peru, with cases occurring regularly in the Iquitos area from 1993 through 2002. Analysis of a larger number of VEEV isolates allowed us to generate a more accurate and complete description of VEE complex circulating in the Amazon Basin of Peru than was possible previously (9). Most human isolates belong to subtype ID, and phylogenetic analyses distinguish two distinct ID genotypes: the Colombia/Venezuela genotype, which is currently circulating in Peru, contrary to previous suggestions (9), and the Panama/Peru genotype, which represents most human isolates. Several hypotheses explain the apparently higher rate of human infection in the Iquitos area by the ID Panama/Peru genotype: 1) viruses from the Colombia/Venezuela genotype are not transmitted efficiently to humans compared to those in the Panama-Peru genotype; 2) the Panama/Peru genotype circulates at higher levels in urban areas such as Iquitos, resulting in more infections; 3) the Panama/Peru genotype causes more severe disease, with more patients visiting clinics and participating in the NMRCD febrile illness study, resulting in more virus isolations and; 4) the Panama/Peru genotype produces higher titer human viremia, resulting in more human isolates. Further ecologic, epidemiologic, and entomologic studies are needed to test these hypotheses. The mosquito vector(s) that transmit VEEV to humans in Peru are unknown. VEEV has been isolated from *Culex* (*Melanoconion*) spp. mosquitoes in Peru, known vectors

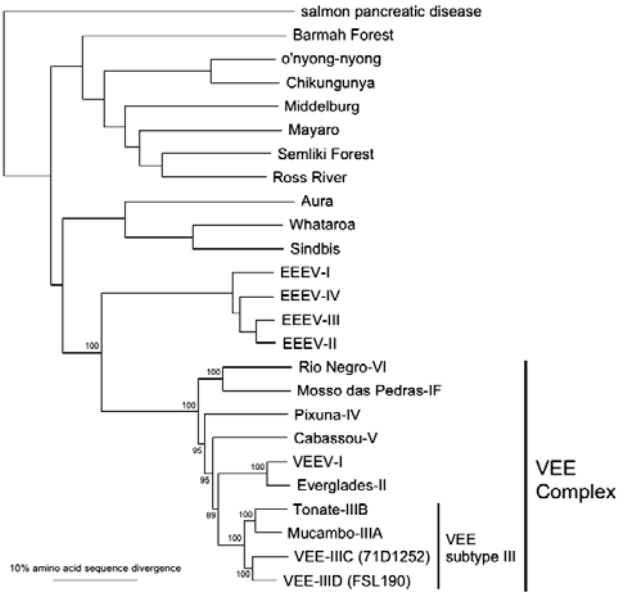

Figure 4. Unrooted phylogenetic tree of the Venezuelan equine encephalitis virus (VEEV) complex and other representative alphaviruses derived from complete structural polyprotein sequences using the neighbor joining program implemented in PAUP 4.0 (22). Viruses are labeled by species according to the International Committee for Taxonomy of Viruses (27). VEEV subtype IIIC and IIID strain names are in parentheses. Numbers indicate bootstrap values for clades to the right.

of enzootic VEEV in many other locations. Several different species (*Culex* [*Mel.*] *gnomatos*, *Cx.* [*Mel.*] *pedroi*, *Cx.* [*Mel.*] *vomerifer*, and *Psorophora albigenu*) are competent laboratory vectors of the ID viruses currently circulating in the Iquitos area (29,30). Further studies are required to determine whether these mosquitoes, other species, or both transmit VEEV to humans in Peru. This information will be important in understanding human exposure and infection with these different VEE complex strains.

Epidemiologic information available from 1995 through 2002 suggests that many VEE cases occurred within the city of Iquitos. Interviews indicated that many patients had not traveled during the time of probable infection, suggesting urban transmission. Studies carried out in other VEEV-enzootic areas indicate that transmission is confined to rural and forest habitats (30–32). Whether the viruses have adapted to infect peridomestic mosquitoes or whether Amazonian deforestation and urbanization is increasing the risk for VEEV transmission to humans through changes in *Cx. Melanoconion* spp. host preference and larval habitats remains to be determined.

Subtype III VEE Complex Strains

Before the strain reported herein, the only isolate of subtype IIIC was obtained in 1971 from a pool of mosquitoes collected near Iquitos (12,13). Although epidemiologic and ecologic studies were conducted in the same area between 1971 and 1995, no evidence was obtained that the IIIC virus was still circulating in the Peruvian Amazon until we isolated it from a sentinel hamster in 2002. The 2002 IIIC isolate did not produce fatal disease in the sentinel animal nor in other hamsters infected experimentally in our laboratory (data not shown). The failure to isolate the subtype IIIC virus from febrile patients in Iquitos suggests that it is not transmitted readily to humans, that the viremia generated in humans is relatively low and usually undetectable by virus isolation, or that the virus simply does not cause clinical human disease.

We propose that the newly identified subtype III strain be defined as a new genetic variety: subtype III, variety D in the VEE complex. These strains do not represent a new antigenic subtype (28) because they exhibit a twofold difference in homologous versus heterologous PRNT antibody titers when compared to the IIIC strain. Arbovirus varieties are described as "isolates differentiable only by the application of special tests or reagents [kinetic hemagglutination inhibition (HI), monoclonal antibody assays, etc.] (28)." We propose that this definition of special tests for variety assignment be extended to include sequence and phylogenetic differentiation of major lineages, such as IIIC versus the proposed IIID genetic variety. These lineages are equivalent in genetic divergence to other VEEV varieties defined previously based on serologic reactions using kinetic HI (8,33).

Both subtype III variants isolated in the Iquitos area are antigenically distinct from Mucambo (IIIA) and Tonate (IIIB) viruses, based on greater than fourfold endpoint antibody titer differences in both directions. The variants are also quite distinct genetically from Mucambo and Tonate viruses (23% nucleotide and 14% amino acid sequence divergence in the structural protein genes) and appear to be geographically distinct as well. We do not have enough information available to know whether the

Table 4. Results of the plaque reduction neutralization tests between members of Venezuelan equine encephalitis subtype III[a]

	Antiserum (subtype/variety)			
Virus strain (subtype/variety)	Mucambo (IIIA)	Tonate (IIIB)	71D1252 (IIIC)	PE407660 (IIID)
Mucambo (IIIA)	1	2	16	8
Tonate (IIIB)	4	1	>160	8
71D1252 (IIIC)	>32	4	1	2
PE4.07660 (IIID)	8	16	2	1

[a]Numbers indicate ratios of homologous to heterologous reciprocal titers.

IIIA, IIIB, IIIC, and IIID variants share vectors or reservoir hosts, although IIIA, IIIC, and IIID probably infect *Proechimys* spp., and Tonate virus (IIIB) may use birds as its reservoir hosts (34).

The newly identified subtype IIID strain was isolated from spiny rats (*Proechimys* spp.), *Culex* (*Melanoconion*) spp. mosquitoes and from a patient with fever, chills, and malaise. The symptoms are typical of human VEEV (subtype I) infection. Using diagnoses based on virus isolation and serology, Watts et al. (4,14) previously reported that VEEV was responsible for at least 3% of febrile illnesses in the city of Iquitos in the Amazon basin of Peru. Subtype ID was thought to be the cause of these human VEEV cases, including those diagnosed only by serologic findings. However, our study demonstrated that both VEEV subtype ID and the newly recognized subtype IIID are responsible for human illness in the Peruvian Amazon basin. Whether subtype IIID was responsible for any other of the 183 human cases diagnosed serologically in Peru since 1995 is unclear. More detailed studies to retrospectively examine serum samples from these cases are required to evaluate this possibility.

Human Disease and Virulence of Enzootic VEEV Strains

In Peru, human VEE does not appear to result in neurologic manifestations, and fatal human disease has never been reported. In contrast, during recent VEEV epidemics with subtype IC strains in Colombia and Venezuela, an estimated 3,000 cases with neurologic complications and 300 fatal cases (5,35) were reported. Overall, human death rates have generally been estimated at approximately 0.5% during these epidemics, with most of the neurologic disease and fatal cases reported in children. Most of the human VEEV cases we studied in Peru occurred in adults (94.6%), which suggests an occupational exposure or possibly an age-biased recruitment into the NMRCD febrile illness study. The lack of any evidence for neurologic disease in any of the NMRCD cases studied from 1994 to 2003 (D. Watts, unpub. data) suggests a possible difference in virulence compared with epizootic IAB and IC strains. Because we characterized only 183 VEEV cases, and only 10 of these were children, whether a virulence difference exists between enzootic subtype ID and epizootic VEEV strains is impossible to determine with any statistical certainty. Strains of the Panama-Peru genotype of subtype ID are known to have caused fatal human disease (10).

Acknowledgments

The authors thank Leslie Angulo, Zonia Rios, Marie Lefevre, and Vidal Felices for their invaluable assistance in the execution of this project.

This work was supported by grant No. AI49725-01 from the NIH, and by Work UNIT NUMBER (WUN) No.847705 8200 25GB B0016. IRB Protocol Number: 31535 “Surveillance and Etiology of Acute Febrile Diseases in Peru.” The study protocol was approved by the Naval Medical Research Center Institutional Review Board (Protocol # 31535) in compliance with all U.S. federal regulations governing the protection of human study participatns. The opinions expressed in this paper are those of the authors and do not reflect the official policy of the Department of Navy, Department of Defense, or the U.S. Government.

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Causative Agent of Pogosta Disease Isolated from Blood and Skin Lesions

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Pogosta disease is a mosquito-borne viral disease in Finland, which is clinically manifested by rash and arthritis; larger outbreaks occur in 7-year intervals. The causative agent of the disease has been suspected of being closely related to Sindbis virus (SINV). We isolated SINV from five patients with acute Pogosta disease during an outbreak in fall 2002 in Finland. One virus strain was recovered from a whole blood sample and four other strains from skin lesions. The etiology of Pogosta disease was confirmed by these first Finnish SINV strains, which also represent the first human SINV isolates from Europe. Phylogenetic analysis indicates that the Finnish SINV strains are closely related to the viral agents that were previously isolated from mosquitoes and that are related clinically similar diseases in nearby geographic areas.

Sindbis virus (SINV), a member of the Western equine encephalomyelitis virus complex of the genus *Alphavirus* in the family *Togaviridae*, was first isolated in 1952 in the Nile River delta in Egypt from a pool of *Culex pipiens* and *Cx. univittatus* mosquitoes (1). SINV is an enveloped virus with a genome of single-stranded, positive-polarity 11.7-kb RNA (2). The genomic 49S RNA also serves as mRNA in the infected cell. Translation of the genomic RNA produces the four nonstructural proteins nsP1–4. The 26S subgenomic mRNA is translated to produce the polyprotein from which E1, E2, and C structural proteins are processed.

The seroprevalence of SINV antibodies among the Finnish population is approximately 2% (3); however, the prevalence varies considerably between different parts of the country. The typical clinical picture of Pogosta disease includes arthritis, maculopapular rash, and sometimes low fever, fatigue, and muscle pain (4; Kurkela et al., unpub. data). Clinically similar or identical diseases are Ockelbo disease (known as August–September disease) in Sweden and Karelian fever in Russian Karelia (5–7). Ockelbo disease was first found in Sweden in 1967 (8). A larger Pogosta disease outbreak has occurred every 7th year in Finland since 1974, with hundreds or thousands of patients (3). The 7-year cycle occurred again in August through September 2002, with 597 serodiagnosed cases reported. The area of highest incidence (8.0/10 000 person-years) was, as in previous epidemic years, the province of North Karelia in eastern Finland, with an incidence almost eight times higher than in the whole country in general. The cause for this geographic distribution is not known. In Sweden, the seroprevalence of SINV antibodies has been highest in the central parts of the country (9). Since all the clinical cases in Finland occur in late July through early October, the virus is most likely carried and transferred to humans by the late summer mosquito species *Culex* and *Culiseta*, from which SINV has previously been isolated in Sweden (10). Possible viral reservoirs of SINV are tetraonid and migratory birds, which are a major blood source for mosquitoes and have been shown to harbor SINV antibodies (3,11,12).

SINV has been isolated from various insects and vertebrates around the world (Figure 1). From a human sample, however, SINV isolation has previously been documented twice. South African Girdwood strain was recovered in 1963 from the vesicle fluid of skin lesions taken from a 45-year-old woman with acute rash-arthritis (13), and Chinese YN87448 strain was isolated from the serum of a febrile patient in 1992 (14). Viral RNA has been detected from skin lesions with the polymerase chain reaction (PCR) method in Sweden (15), but no genetic sequence is available. No virus isolations or detections have previously taken place in Finland. The aim of this study was to isolate the causative agent of Pogosta disease directly from human samples.

Patients and Methods

Clinical Samples

We collected samples from the health districts where the incidence of Pogosta disease had been highest during previous epidemic years, North Karelia and Kuopio. When acute SINV infection was suspected on clinical examina-

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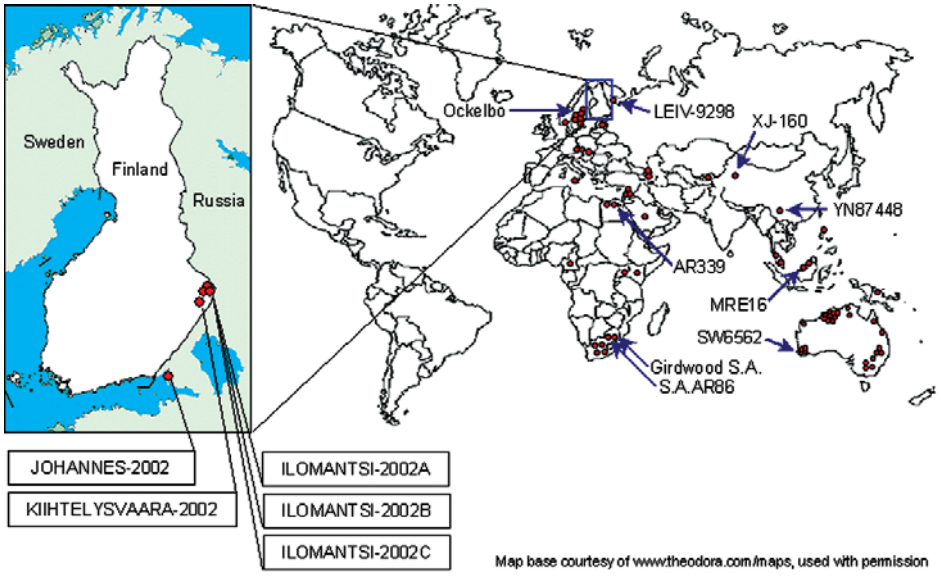

Figure 1. Sindbis virus isolates around the world. Each dot represents either one strain (isolated from insect or vertebrate) or several strains isolated from specific *Diptera* genus (e.g., *Culex* or *Aedes*) at the same time and place. The strains included in the phylogenetic analyses are indicated with arrows. The enlarged map presents the new Sindbis virus isolates introduced in this study.

tion, whole blood samples in tubes containing ethane diamine tetraacetic acid (EDTA) as anticoagulant and skin lesion biopsy specimens were collected, if possible. The skin biopsy specimens were taken from one papulopustule with a punch or a surgical knife into a dry tube, usually by a local physician. The samples were kept at 4°C, and transported to the local central hospital usually within the next 24 hours to be frozen at –70°C. All samples were kept frozen until they were processed at the department of Virology, University of Helsinki. Informed consent was obtained from all patients. The study was carried out under the permission of the coordinating ethical committee of the Hospital District of Helsinki and Uusimaa.

Representative Case Report

A 35-year-old man was most probably exposed to SINV in August 2002, in Ilomantsi, province of North Karelia. Typical Pogosta disease symptoms began with polyarthritis, first affecting the fingers, wrists, and the left shoulder, then extending to the left elbow, and finally both knees and the right ankle. Joint symptoms were aggravated by exertion. At the acute phase, the patient also had fatigue, nausea, muscle pain, headache, low fever, and itchy rash throughout the body. A skin biopsy was taken from one papulopustule (see also Table; patient 1). Basic blood parameters at the acute phase were within normal range, including blood cell counts, hemoglobin, platelets, and the erythrocyte count indices. The joint symptoms lasted for 3 to 4 months. No other residual symptoms were encountered.

Serodiagnostic Methods

All patients were screened for SINV immunoglobulin (Ig) M and IgG with enzyme immunoassay (EIA) and for total antibodies with the hemagglutination inhibition test (HI). EIA was performed with purified SINV antigen, directly coated on microtiter well plates (16). For HI microtitration, serum samples were absorbed with kaolin and male goose erythrocytes and tested against SINV (grown in BHK21/WI-2 cell monolayers) and 0.2% suspension of goose erythrocytes (3). For the diagnosis, either a positive result in EIA for SINV IgM in a single sample or seroconversion (or greater than fourfold increase in titer) between paired serum specimens was required.

Table. Samples from which Sindbis virus (SINV) was isolated[a,b]

Patient	Strain	Sample	Sex	Age (y)	Probable place of exposure	Onset of symptoms	Disease/rash days at time of sampling	SINV IgM status at time of sampling
1	Ilomantsi-2002A	Skin lesion	M	35	Ilomantsi, Finland	8/ 22/2002	3/ 1	Negative
2	Ilomantsi-2002B	Skin lesion	M	30	Ilomantsi, Finland	8/18/2002	2/ 0	Negative
3	Ilomantsi-2002C	Whole blood	F	47	Ilomantsi, Finland	Late Aug. 2002	N/A	Borderline
4	Johannes-2002	Skin lesion	M	63	Johannes, Russia	8/27/2002	9/7	N/A
5	Kiihtelysvaara-2002	Skin lesion	M	39	Kiihtelysvaara, Finland	8/30/2002	3/ 2	Negative

[a]The places of exposure are shown on the map (Figure 1). The representative case report given in the text describes the patient from whom the Ilomantsi-2002A virus strain was recovered.
[b]M, male; F, female; N/A, not available; Ig, immunoglobulin.

Virus Isolation

The frozen skin samples were cut into small pieces, then homogenized in a mortar, and suspended in 100–150 µL Dulbecco's minimal essential medium plus 0.2% bovine serum albumin. A total of 50–100 µL of this suspension diluted in 500 µL of culture medium, containing minimal essential medium and 2% fetal calf serum with a mixture of glutamine, ampicillin, and penicillin, was added to confluent Vero cells in 25-cm^2 cell culture flasks. Whole blood samples were diluted 1:10 in culture medium, and confluent Vero cell cultures were injected with the final volume of 500 µL. The cells were incubated for 1 h at 37°C, then 3–4 mL culture medium was added, and the cultures were kept at 37°C. The toxicity of EDTA-anticoagulant on Vero cells could be avoided by completely removing the blood dilution from cells after the 1-h incubation and also by changing the culture medium the day after the injection and then twice a week. All cell cultures were inspected daily. When cytopathic effect (CPE) was apparent, immunofluorescence assay (IFA) was performed, and the cells were passaged.

Several measures were undertaken to avoid laboratory contamination. SINV was not handled in the two laboratories where the samples were prepared and the cells were cultured, and separate sets of instruments were always used. Samples were handled and cells cultured in separate laboratories. Cross-contamination was avoided by culturing in cell culture flasks instead of culture plates. At least two flasks of noninfected Vero cells were cultured in the same incubator every time and went through the same procedures as the potentially infected cells.

IFA

An IFA was developed to confirm SINV infection of the cells. Cells were harvested in 600 µL phosphate-buffered saline (PBS), washed, and centrifuged at 1,800 rpm for 3 min five times and dried on a slide. For immunofluorescence staining the slides were fixed for 7 min in ice-cold acetone. A pool of 10 SINV IgG–positive serum samples were diluted 1:20 in PBS, added to slides, and incubated in a moist chamber at 37°C for 30 min. The slides were washed three times in PBS and once in water, then incubated at 37°C for 30 min with fluorescein isothiocyanate–conjugated F(ab')2 goat anti-human IgG diluted 1:100 in PBS. After another wash, the slides were dried, mounted, and screened with a fluorescence microscope.

RNA Extraction and Reverse Transcription (RT)-PCR

Viral RNA was extracted from culture supernatant with TriPure Isolation Reagent (Roche Molecular Biochemicals, Espoo,) by following the manufacturer's instructions. The primers used in RT-PCR were 5′-TTTAGCGGATCGGACAATTC-3′ and 5′-GCGGTGACGAACTCAGTAG-3′. The RT reaction was carried out as follows: 10 µL of RNA dissolved in water was mixed with 2 µL of each primer (10 pmol/µL), 2 µL of M-MuLV reverse transcriptase (20 U/µL [MBI Fermentas, Vilnius, Lithuania]), 5 µL of 5 x RT-buffer (MBI Fermentas), 2 µL of dNTP-mix (2.5 mol/L dATP, dTTP, dGTP, and dCTP [Finnzymes, Espoo, Finland]), and 2 µL of Ribonuclease inhibitor (40 U/µL; MBI Fermentas). The mixture was incubated at 37°C for 90 min.

For the PCR 7 µL of cDNA were incubated at 95°C for 5 min, cooled immediately on ice, and mixed with 2 µL of each primer (10 pmol/µL), 10 µL of 10 x Taq Extender PCR Buffer (Stratagene, La Jolla, CA), 8 µL of dNTP mix (2.5 mM; Finnzymes), 1 µL of Taq DNA Polymerase (recombinant) (5 U/µL; MBI Fermentas), 1 µL of Taq Extender PCR Additive (Stratagene), and 69 µL of water. The reactions were amplified through 35 cycles using a DNA thermal cycler with the following steps: denaturation at 95°C for 45 s, annealing at 55°C for 2 min, and elongation at 72°C for 3 min, followed by a final extension at 72°C for 10 min.

Cloning

The PCR amplicons were purified with QIAquick gel extraction kit (Qiagen, Hilden, Germany). The amplicons were cloned with TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA), following the manufacturer's instructions, and transformed into TOP10 chemically competent *Escherichia coli* cells on bacterial plates containing x-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (isopropylthio-β-D-galactoside) for blue-white screening. The plasmid DNA was isolated with QIAprep Miniprep kit (Qiagen). and restriction analysis was performed. Vector-based primers M13 Reverse and T7 were used for automatic sequencing with ABI PRISM (Perkin-Elmer, Foster City, CA).

Sequence Analysis and Phylogenetic Analysis

Sequences were aligned with Clustal W1.75 program (17) into MSF-format and edited with GeneDoc Multiple Sequence Alignment (available from http://www.psc.edu/biomed/genedoc/) editor program. Sequence was confirmed from at least three different clones. PHYLIP program package (Felsenstein, 1993) was used to create 5,000 bootstrap replicates on the sequence data (SEQBOOT). Distance matrices were calculated with DNADIST program with Kimura's two-parameter model of substitutions and analyzed by neighbor-joining tree-fitting algorithm with NEIGHBOR program. The bootstrap support values were calculated with CONSENSE program.

Results

Altogether 131 patients with suspected acute Pogosta

disease were recruited to the study in 11 different health stations in the province of North Karelia and in Kuopio University Hospital, Finland, during July through October of 2002. A total of 86 patients had acute, serologically confirmed SINV infection. Twenty-three skin biopsy specimens and 73 whole-blood samples (treated with EDTA) were collected from patients with acute SINV infection and used in this study for isolation attempts.

A physician examined all patients at the acute phase. To determine the most likely place and time of exposure to SINV, background information about the physical location of the patients before the onset of symptoms was also collected by using questionnaires. The exact incubation period in Pogosta disease is unknown, and determining the time and place of exposure was difficult.

Virus isolation was successful from 4 of 23 skin samples and 1 of 73 whole blood samples (Table) (Figure 1). All isolates induced a strong CPE in cell culture, featuring round-shaped, interconnected cells and cell death within 72 hours of culturing. Infected cells from all isolates were shown to give a strong positive signal with IFA with human SINV IgG–positive sera. All noncytopathic cell cultures were also screened and found negative with IFA, e.g., no discrepancy between the isolation method and immunofluorescence staining was encountered.

The median and average number of days of rash for patients with the positive skin samples before sampling were 1.5 and 2.5, respectively, and SINV IgM antibodies were not detectable (IgM status for patient 4 at the time of sampling was not available) (Table). The median and average days of rash for the negative skin samples until sampling were 2.0 and 1.7, respectively (data available from 17 of 19 patients). In all, 37% of the patients who gave a skin biopsy sample had detectable SINV IgM antibodies: 16% had a borderline result at the time of sampling (data available from 19 of 23 patients). The median and average number of disease days before collecting the whole blood samples were 3.0 and 4.5, respectively (data available from 65 of 73 patients). The only blood sample from which SINV could be isolated had a borderline SINV IgM result; the exact time of onset of symptoms is not available for this case.

The nucleotide sequences of 1,178–1,281 bp from nsP3 and nsP4 region of the new strains were determined for the phylogenetic analysis and submitted to GenBank. The strains were given the following accession numbers: Ilomantsi-2002A (AY532322), Ilomantsi-2002B (AY 532326), Ilomantsi-2002C (AY532324), Kiihtelysvaara-2002 (AY532325), and Johannes-2002 (AY532323). In addition, we sequenced from this region the LEIV-9298-strain (AY532321), isolated from *Aedes* mosquitoes in 1983 in central Russian Karelia, approximately 200 km north of Ilomantsi, Finland (6). The following sequences available in GenBank were included in the comparison: AR339 (HRsp variant), Girdwood S.A., MRE16, Ockelbo (Edsbyn 82), S.A.AR86, SW6562, YN87448, and XJ-160. See Figure 1 for the geographic location and Figure 2 for the phylogenetic tree of the strains. Sequence comparisons and phylogenetic analysis show that the northern European (e.g., Finnish, Russian, and Swedish) SINV strains analyzed in this study are closely related, with a percentage difference of 0.1% to 1.4% on nucleotides and 0% to 2.1% on amino acids. The Russian Karelian LEIV-9298 and Johannes differ by one nucleic acid, and their amino acid sequences are identical. Malaysian MRE16 is furthest from Finnish strains when both nucleic and amino acids are compared, differences are 35.6% to 35.8% and 28.3% to 28.5%, respectively.

Discussion

This study describes the first human SINV isolates from Europe, one strain from blood and four from skin

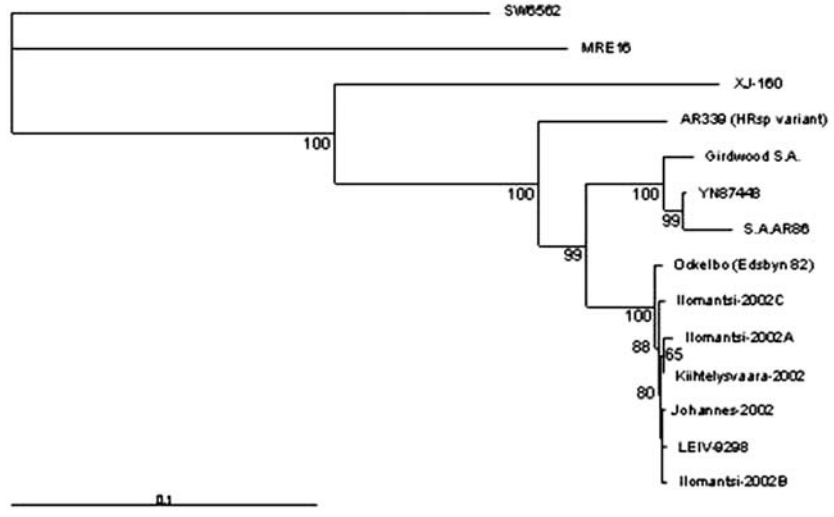

Figure 2. Phylogenetic tree is based on the nucleotide sequences of 1,178–1,281 bp from nsP3 and nsP4 region, nucleotides 5,258-6,510; the genome position is given according to the published sequence of the strain AR339 (HRsp variant) (2). The tree was constructed by using neighbor-joining algorithms (NEIGHBOR). 5,000 bootstrap replicates were calculated. Only those bootstrap support values that exceed 50% are shown. The following sequences available in GenBank were included in the comparison: AR339 (HRsp variant); Egypt (J02363, J02364, J02365, J02366, J02367), Girdwood S.A.; South-Africa (U38304), MRE16; Malaysia (AF492770), Ockelbo (Edsbyn 82); Sweden (M69205), S.A.AR86; South-Africa (U38305), SW6562; Australia (AF429428), YN87448; China (AF103734) and XJ-160; China (AF103728).

lesions. One of the strains is apparently Russian, since the Finnish patient most likely was exposed to SINV in Johannes (Sovetskiy), Russian Karelia. The four other strains represent the first SINV isolates from Finland. Phylogenetic analysis of the strains shows a close relationship to Swedish and Russian SINV strains, isolated approximately 20 years ago from mosquitoes.

The possibility of laboratory contamination was minimized by various measures as described in Methods. Only a few samples were prepared at the same time; each new virus strain was isolated from separate set of samples. No CPE was apparent in the negative control cells at any stage and they were all negative in immunofluorescence staining as well.

SINV could be recovered from one blood sample of 73. This sample was positive by nested RT-PCR method as well; full characterization of clinical and laboratory data will be presented later (Kurkela et al., unpub. data). As now proven, SINV is present in the blood during acute infection. The most viremic window appears to be very narrow and the level of viremia can vary considerably between persons. These presumptions make future laboratory diagnostics based on viral detection challenging, and serology likely will remain the method of choice for diagnosis.

In skin tissue, the viral persistence seems to last for several days, if not weeks. The Johannes-2002 strain was isolated from a biopsy specimen taken 7 days after the onset of rash. SINV could be recovered from 17% of the skin biopsy samples. Whether the virus persists in synovial fluid and whether this could explain the prolonged joint symptoms in a substantial proportion of Pogosta disease patients (Kurkela et al., unpub. data) remain to be determined. Some patients have had borderline results in IgM serologic testing even months or years after the onset of disease (18), although no correlation between prolonged joint symptoms and elevated IgM levels in serum has been observed. In experiments with mice, evidence indicates that viral replication can take place in the periosteum or endosteum (19). Efforts to detect SINV or SINV RNA from human synovial fluids have failed (20). Clinical indications to retrieve synovial fluid samples rarely occur in Pogosta disease, and therefore no synovial fluids were available in this study.

The cell type in which the virus replicates in skin tissue is not known. In histopathologic examination of skin lesions of Pogosta disease patients, a pronounced lymphohistiocytic inflammatory infiltrate and lymphoblast-like cells have been described (21). Since the virus is present in the skin during acute infection before the onset of antibody response (Table), the cutaneous manifestations in Pogosta disease may be due to a direct viral effect; however, a more complex immunologic reaction behind the pathogenesis can certainly not be excluded.

Four patients were serodiagnosed with acute SINV infection in Sweden during the Finnish outbreak in 2002 (Sirkka Vene, pers. comm.), which suggests that local ecologic and environmental factors are involved in the epidemiology of Pogosta disease in Finland. Data are not available about whether the epidemics in northern Karelia were accompanied by similar epidemics on the Russian side of the Finnish-Russian border, although one of our patients (Table; patient 4) was most probably exposed while traveling in Russian Karelia. Ecologic circumstances likely alter the viral cycle in nature, and the prevalence of mosquitoes carrying SINV varies. However, differences in the pathogenicity of viral strains cannot be excluded. In Australia, for instance, SINV is the most common arbovirus isolated from mosquitoes , but human infections have often been either subclinical or mild (23,24,25). Furthermore, virus isolations from different animals have also taken place in central Europe (26) without any reported human cases.

Phylogenetic analysis of the Finnish SINV strains for a 1.2-kb genome segment indicates that Finnish SINV strains are closely related to each other and to the Swedish and Russian strains, isolated 2 decades ago (Figure 2). Within this genome segment, Johannes-2002 strain is almost identical to LEIV-9298 strain, isolated 20 years earlier, ≈500 km north of Johannes. Northern European SINV strains might constitute a restricted geographic area of viral emergence, which suggests that the virus is maintained locally in disease-endemic regions. However, viral redistribution over long distances is possible, which would introduce new, distinct SINV strains to northern Europe. Viral recombination could also account for further variation, but more sequence information from other gene regions and SINV strains is required to define its role.

South African SINV strains have been shown to not vary substantially from Swedish strains (26). Therefore, the question still remains how and from where the viral importation to Finland has taken place. One possibility could be through mosquitoes carried by air currents. Migratory birds would be plausible viral carriers from further distances, such as Africa. Up to 50% of migratory and tetraonid birds have been shown to carry SINV antibodies in Finland (3); birds could function both as viral importers and amplifying hosts (11,12). Resident and migratory birds have recently been shown to carry SINV antibodies also in the United Kingdom (27). The phylogenetic analysis in this study supports this concept by suggesting that redistribution of SINV tends to occur in a longitudinal, not latitudinal, direction. Contrary to the above, the strain YN87448, which originates from eastern China, is positioned in the phylogenetic tree close to the African strains (Figure 1). Indications of periodic redistribution of SINV strains over long distances and within a short time have

previously been demonstrated in Australia (28), a finding consistent with the involvement of migratory birds. SINV could be frequently introduced to Finland or hide locally through winter in some natural reservoir, to emerge again in August through September. The available data favor maintenance of SINV in a local endemic cycle but the extent to which new introductions of SINV may play a role remains a subject for further investigation.

Acknowledgments

We thank the staff at North Karelian health stations involved in material collection; Suvi-Sirkku Kaukoranta and Camilla Jokinen for essential organizing support; Kimmo Linnavuori for expert advice regarding cell culture experiments; and Sirkka Vene for important information and for providing the LEIV-9298 virus strain.

This study was supported by an EVO grant from the Hospital District of Helsinki and Uusimaa (TYH2277).

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Infection Control and SARS Transmission among Healthcare Workers, Taiwan

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This study found infrequent transmission of severe acute respiratory syndrome (SARS) coronavirus to healthcare workers involved in the care of the first five case-patients in Taiwan, despite a substantial number of unprotected exposures. Nonetheless, given that SARS has been highly transmissible on some occasions, we still recommend strict precautions.

Healthcare workers may be unwittingly exposed to the severe acute respiratory syndrome–associated coronavirus (SARS-CoV) from patients with pneumonia at the onset of an epidemic (1,2). They are also at increased risk of acquiring SARS from known case-patients with a high viral load who require intensive respiratory care (1–3). The first case-patient in Taiwan was admitted to National Taiwan University Hospital on March 8, 2003, before the World Health Organization (WHO) issued the first global alert (4,5). The patient was intubated in the emergency room and was admitted to the intensive care unit. The second case-patient, his wife, was admitted to the emergency room with pneumonia on March 14. The occurrence of two cases of pneumonia in the same household within 6 days, together with the patients' recent travel to Guangdong, China, through Hong Kong, led us to suspect a diagnosis of atypical pneumonia, which later came to be known as SARS.

Before the second case was detected, healthcare workers routinely used standard precautions. Specific infection-control measures, including droplet and contact precautions against SARS, were implemented after the second patient was admitted. The efficacy of these infection-control measures in protecting healthcare workers was determined by: 1) the occurrence of SARS symptoms as defined by WHO criteria (6) and 2) a rise in antibodies to SARS-CoV before and after specific infection-control measures were implemented.

The Study

From March 8 to March 28, the hospital admitted five patients in whom SARS-CoV infection was subsequently laboratory-confirmed. The patients were isolated in negative-pressure rooms. Patients 2 and 3 were family members of patient 1. Patients 4 and 5 were believed to have contracted SARS on a March 15 flight from Hong Kong to Beijing. Four of the patients progressed rapidly to respiratory failure and were intubated.

Healthcare workers caring for these patients were exposed during two periods. During March 8–14, before specific infection-control precautions were implemented, 73 healthcare workers were exposed to patients 1 and 2. During March 15–28, after specific precautions were implemented, an additional 150 healthcare workers were exposed to all five patients.

All healthcare workers who had contact with SARS patients used personal protective equipment, including gown, gloves, N95 respirators, disposable cap, and shoe covers. Healthcare workers exposed to SARS patients or their environments were monitored for signs or symptoms of SARS for 14 days after the last exposure. Healthcare workers who had high-risk exposures to SARS were excluded from new duty assignments. We considered performing any of the following to be a high-risk exposure: endotracheal intubation >30 min, cardiopulmonary resuscitation >30 min, pleurocentesis >30 min, or bedside care (such as chest care [including percussion and postual drainage] or feeding) >30 min. Any healthcare worker in whom fever developed (temperature ≥38°C) was isolated in a specially designated ward.

A total of 223 healthcare workers exposed to SARS patients were interviewed by one of two researchers with a structured questionnaire designed by the Centers for Disease Control and Prevention (CDC), USA, and the Center for Disease Control, Taiwan. The following data were recorded on uniform case-report sheets: extent of personal protective equipment use during exposure, type of exposure (stay in the same room, direct patient contact, or exposure to respiratory droplets and secretions), disease phase of patients to whom they were exposed (during incubation period, early fever, fever and cough, or intubation period), occurrence of fever (≥38°C), and respiratory or

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gastrointestinal symptoms after exposure. Proportional data were tested by using χ^2 or Fisher exact test (EpiInfo 6, CDC, Atlanta, GA). A p value <0.05 was considered significant. The Ethics Committee of the hospital approved these studies.

Serum samples were collected twice from 206 healthcare workers during a 1-month period after the initial exposure to patients with SARS, with a minimum interval between collections of 2 weeks. Serologic response to SARS-CoV was determined by using an indirect immunofluorescence assay (IFA) as described previously (5) and the immunochromatographic test (ICT, Tyson BioResearch, Inc, Taiwan). ICT consists of a double-antigen (recombinant viral nucleocapsid antigen) sandwich. The test gives results within 15 min. Data obtained from 13 patients with severe SARS, as defined by using CDC criteria (7), showed that the sensitivity of the ICT test was >90% within 2 weeks of fever onset and 100% after 6 weeks. Data obtained from 51 cases of severe SARS demonstrated that the sensitivity of either IFA or ICT was 98% after 6 weeks (8). Furthermore, the specificity of each assay determined by 812 serum samples was 100%.

The Table compares the extent of personal protective equipment use before and after implementing specific infection-control measures. Healthcare workers during the "after" period were substantially more likely than the "before" period to have used full personal protective equipment (Table).

First serum samples were collected 12.4 ± 5.4 days (mean ± standard deviation) after initial exposure to SARS patients. Second serum samples were collected 37.2 ± 7.9 days after exposure. Ninety percent were collected ≥30 days after exposure. None of the 73 healthcare workers exposed during the before period produced a positive result on serologic tests for SARS. This group included a physician who intubated patient 1 and wore two layers of surgical masks and used inline suction after intubation. SARS developed in 1 of 150 healthcare workers exposed during the after period. This healthcare worker was a chest physician. On March 17, he performed a 30-min chest sonogram on patient 2 in a negative-pressure isolation room and wore an N-95 respirator, double gloves, gown, disposable cap, and shoe covers. On the same day, he helped intubate patient 2 while positioned approximately 3 feet from the patient's head. During this period, patient 2 was irritable and had a vigorous cough. The physician recalled that he had not tried the mask on or confirmed that it was air-tight before entering the isolation room. Fever developed 4 days later in this physician, designated as patient 6, and pneumonia developed 5 days after that. Both virus culture and reverse transcriptase–polymerase chain reaction (RT-PCR) demonstrated SARS-CoV in the sputum. Immunoglobulin (Ig) G against SARS-CoV determined by IFA was >1:1,000 (5). After this experience, the infection-control team reemphasized the importance of fit-testing facemasks and recommended wearing a face shield when in close contact with SARS patients. SARS did not develop in another physician who intubated patient 2 and four nurses who assisted the procedure in the same room.

Conclusions

In this study, a physician who intubated a patient with SARS while following standard precautions did not become ill, but SARS developed in another physician whose N95 respirator was not properly fit-tested. A serologic response to SARS-CoV could not be demonstrated in

Table. Personal protection before and after recognizing severe acute respiratory syndrome (SARS) and implementing specific infection-control measures at the National Taiwan University Hospital

	Exposure type								
	In the same room[a]			Direct contact			Exposure to respiratory droplets and secretions		
Protective measures	Before (n = 73)	After (n =155)	p value	Before (n = 46)	After (n = 132)	p value	Before (n = 37)	After (n = 92)	p value
Masks			<0.001			<0.001			<0.001
None	36	0		20	0		17	0	
Surgical mask, N95 or P100 respirator	37	155		26	132		20	92	
Gloves			<0.001			<0.001			<0.001
None	57	7		28	4		17	2	
One- or two-layer	16	148		18	128		20	90	
Eye protection			<0.001			<0.001			<0.001
None	73	117		46	99		37	66	
Glasses, goggles, or face shields	0	38		0	33		0	26	
Gowns			<0.001			<0.001			<0.001
None	66	6		38	6		30	3	
One- or two-layer	7	149		8	126		7	89	

[a]Five healthcare workers stayed in the same room with SARS patients before and after implementation of specific infection-control measures. Among 223 healthcare workers, 178 had direct contract to SARS patients or their environment, and 129 had exposure to respiratory droplets and secretions.

205 healthcare workers who spent time in the same room as or had direct contact with SARS patients.

The major question that arises from this study is why 36 (50%) healthcare workers who stayed in the same room with SARS patients before the outbreak was recognized and who did not wear masks were not infected. Several possible explanations exist. Patient 2 wore a face mask when she visited the emergency room. The physician who intubated patient 1 was alert, wore two layers of surgical masks, and followed standard precautions. Inline suction was routinely performed at the hospital for intubated patients to prevent aerosol formation; therefore, unprotected healthcare workers might not have been exposed to a sufficient amount of SARS-CoV to produce a systemic infection. An alternate explanation could be that existing serologic assays are not sufficiently sensitive to identify subclinical infections. This explanation is unlikely, however, because the tests we used have been shown to be highly sensitive and specific in patients with SARS (5,8), and 90% of convalescent-phase serum samples were collected ≥30 days after exposure. Yet another explanation could be that SARS-CoV is attenuated by serial passage in humans. This explanation is also unlikely since SARS developed in the five index patients admitted to the hospital in the early phase of the epidemic and in one physician with a poorly fitting mask. Further, phylogenetic tree analysis (9) indicates that patients 2, 3, and 6 were infected by strains related to the large outbreak in Amoy Gardens in Hong Kong (2), and patients 4 and 5 were infected by strains related to a large hospital outbreak in Taipei (10). A final explanation could be, simply, that the disease does not develop in all people exposed to the virus.

Transmission of SARS was limited initially at our hospital (attack rate 0.4%) when healthcare workers followed standard precautions or specific infection-control measures, including droplet and contact precautions. However, in later stages of the epidemic, SARS was more likely to develop in healthcare workers, despite similar or higher levels of personal protective equipment use. Although one possible explanation for this could have been exposure to unrecognized SARS patients, contamination of the environment leading to indirect contact transmission may have also played a role (11).

In conclusion, while SARS-CoV can spread rapidly in a nonimmune human population (1–3), this study demonstrated infrequent transmission of SARS to healthcare workers caring for the first five SARS patients in Taiwan, despite a number of unprotected exposures. Nonetheless, given that SARS has, on other occasions, shown itself to be highly transmissible (1–3,10), we still recommend strict precautions (1–3,11–14).

Acknowledgments

We are grateful to members of the infection-control team for their important contributions to this investigation and Calvin Kunin for his critical review of this manuscript.

This study was supported by a grant from the National Science Council, R.O.C. (NSC 92-3112-B-002-043).

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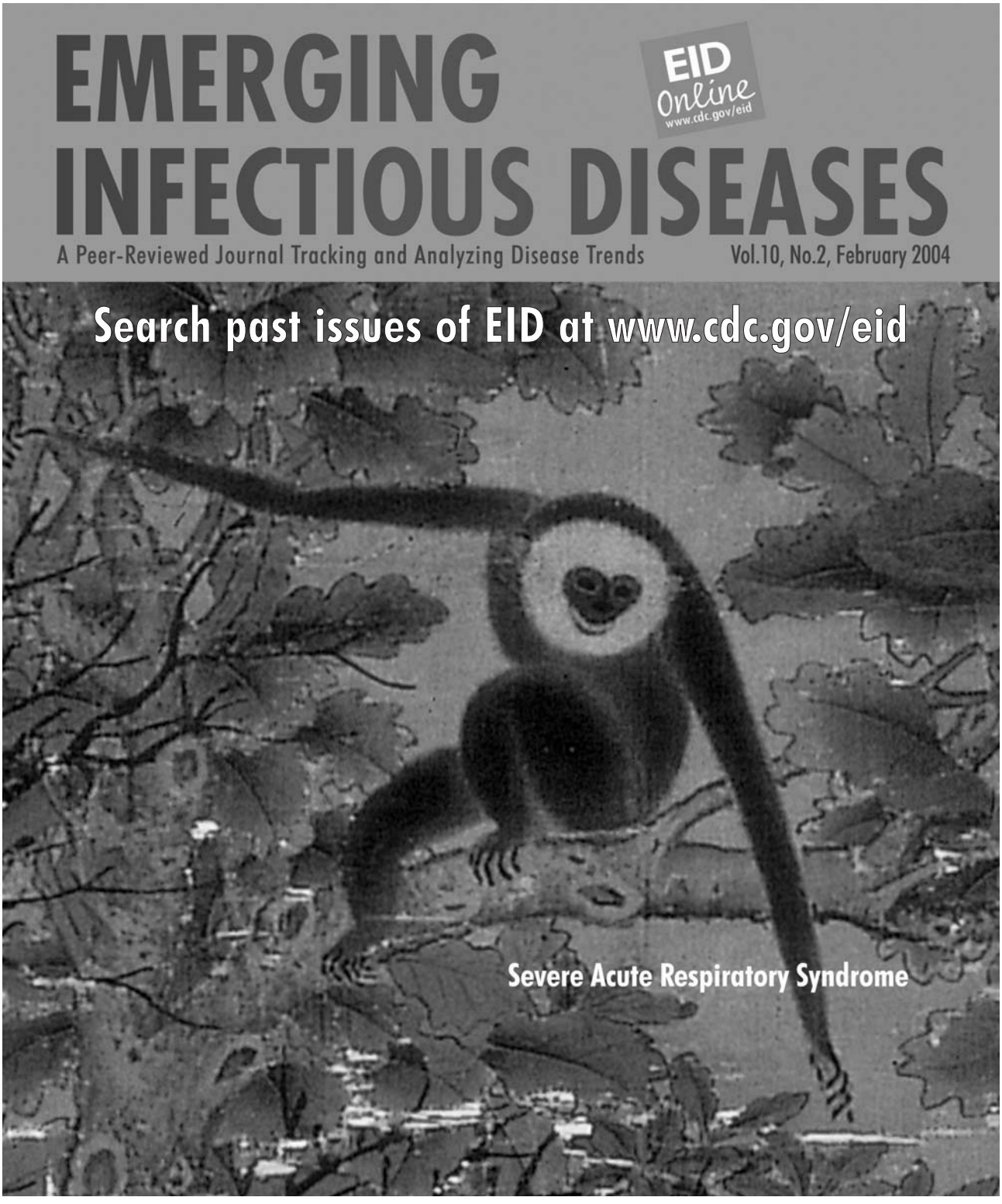

Novel γ-2-Herpesvirus of the *Rhadinovirus* 2 Lineage in Gibbons

Renan Duprez,* Emmanuelle Boulanger,* Yannick Roman,† and Antoine Gessain*

We obtained 475 nucleotides of the DNA polymerase gene of a novel human herpesvirus 8 homolog sequence in a gibbon. The finding of this new gibbon virus, which clusters with a related chimpanzee virus in the rhadinovirus 2 genogroup, suggests the existence of a novel γ-2-herpesvirus in humans.

Among *Gammaherpesvirinae*, Epstein-Barr virus (EBV) and Kaposi sarcoma–associated herpesvirus or human herpesvirus 8 (KSHV/HHV-8) represent human prototypes of the *Lymphocryptovirus* (γ-1-herpesvirus) and *Rhadinovirus* (γ-2-herpesvirus) genuses, respectively (1). Both viruses play an important role in human multistep carcinogenesis, especially in immunodeficient patients. Indeed, Kaposi sarcoma and EBV-associated Burkitt lymphoma currently represent the most frequent cancers in some geographic areas, especially in Africa.

Several γ-1-herpesviruses have been isolated from nonhuman primates, especially African and Asian apes, since the 1970s (2,3). However, most of the sequences of these viruses, which allow phylogenetic studies, have only been recently obtained (4).

Rhadinoviruses (or γ-2-herpesviruses) have also been found in many animal species including New and Old World primates. Among the latter, recent comparison and phylogenetic analyses of all available sequences support the existence of two distinct genogroups called RV1 and RV2 for Rhadinovirus genogroups 1 and 2 (5–9).

Among African great apes, four γ-2-herpesviruses have recently been discovered. These are PanRHV1a/PtRV1 (Pan rhadinovirus 1a/*Pan troglodytes* rhadinovirus 1), PanRHV1b (Pan rhadinovirus 1b) and GorRHV1 (gorilla rhadinovirus 1) from chimpanzees and gorillas respectively, in the RV1 group and PanRHV2 (Pan rhadinovirus 2) from chimpanzees in the RV2 group (7,10,11). By contrast, γ-2-herpesvirus has never been found, so far, in Asian apes. The goal of this study was therefore to search for γ-2-herpesviruses in Asian apes.

To look for KSHV-related viruses, we first performed a serologic analysis. Plasma of 38 captive Asian apes, including 30 orangutans (25 *Pongo pygmaeus pygmaeus* and 5 *P. p. abelii*) and 8 gibbons (including 1 *Hylobates gabriellae* and 7 *H. leucogenys*), originating from different zoos and primate centers mainly from France, were tested by two different immunofluorescence assays, as previously described (12,13). Briefly, the first method, which uses KSHV-infected human KS-1 cells as source of antigens, allows mainly the detection of antibodies directed against lytic KSHV antigens, while the second, using KSHV-infected BC3 cells, allows only for the detection of antibodies directed against the latent nuclear antigen. Results demonstrated a clear antilytic cross-seroreactivity in plasma samples from 10 orangutans and 3 gibbons with titers from 1/20 to 1/160, while seroreactivity against the latent nuclear antigen was more rarely detected (7 orangutans and 2 gibbons), mostly with very faint patterns.

We then performed a polymerase chain reaction (PCR)-based study using high molecular weight DNA extracted either from peripheral blood mononuclear cells or from buffy-coat of 35 of these Asian great apes (8 gibbons and 27 orangutans for which DNA was available). We attempted to amplify a small fragment of the highly conserved herpesvirus DNA polymerase gene by heminested PCR, using previously described consensus-degenerate primers (7,8,14). Among the 35 DNA samples, 18 scored positive (5 gibbons and 13 orangutans) on the ethidium bromide gel, with a band of the expected size (237 bp), corresponding to the KSHV-positive control. Purifying, cloning, and sequencing of these products indicated the presence, in 8 orangutans and 4 gibbons, of two species-specific lymphocryptovirus sequences, that were slightly different from each other but very closely related to EBV, as recently reported (4). However, in 1 *H. leucogenys* (gibbon 7), database searches, using BLAST web server (available from http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), indicated a novel γ-herpesviral DNA polymerase sequence that was closely related to the *Rhadinovirus* genus strains. No other novel herpesviral sequence was detected in orangutans or gibbons.

Using the same PCR approach with a specific reverse primer for the second PCR (HyloRHVas: CAT CGT GCG TCC CTG CAG CG), we amplified, from the DNA sample of gibbon 7, a second overlapping fragment, resulting in a 475-bp final fragment of a new herpesviral DNA polymerase gene (GenBank accession no. AY465375). Nucleotide comparison of a 451-bp fragment, corresponding to the best alignment of all the γ-herpesviruses available sequences, indicated that the novel gibbon rhadinovirus sequence was more closely related to the corresponding sequences of the RV2 genogroup viruses (76%, 73%, and 71% of nucleotide identity with ChRV2 (Chlorocebus rhadinovirus 2), MndRHV2 (Mandrillus

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rhadinovirus 2) and PanRHV2, respectively), than to the corresponding sequences of the RV1 genogroup viruses (70%, 69 %, and 63% of nucleotide identity with KSHV, PanRHV1a, and PanRHV1b fragments, respectively). Similar results were obtained when the comparison was done on the amino acid sequences.

Phylogenetic analyses, by using two different methods (neighbor joining and DNA maximum parsimony), were performed with two different sets of sequences. The first set comprises all available primate γ-herpesvirus DNA polymerase gene sequences. The second set comprises most of the available corresponding herpesvirus sequences, including the γ-2-herpesviruses originating from nonprimates species. Nearly identical tree topologies were obtained for the two phylogenetic methods when the analyses were restricted to the primate sequences (Figure 1; data not shown). The addition of the γ-2-nonprimate herpesviruses (Figure 2) modified the positioning of some sequences within the γ-2 herpesviruses and some bootstrap values as compared to the analysis restricted to primate viruses. However, all analyses clearly localized, with bootstrap values from 83% to 85%, the novel gibbon viral sequence (HyloRHV2) within the *Rhadinovirus* genus in the RV2 genogroup (Figures 1 and 2; data not shown). All together, these studies as well as previous works, demonstrate the existence, among the primate γ-2-herpesviruses, of three distinct separate lineages, as seen on Figure 1. The first lineage corresponds to the New World group, including rhadinoviruses of spider and squirrel monkeys. The second lineage, the RV1 group, comprises the rhadinoviruses of Old World primates, including those of humans, chimpanzees, gorillas, African green monkeys, mandrills, and macaques. The third lineage, which corresponds to the RV2 group, also contains rhadinoviruses of Old World nonhuman primates (chimpanzees, African green monkeys, macaques, baboons, mandrills, and our novel gibbon HyloRHV2). The novel HyloRHV2 sequence clearly localized with the PanRHV2 strain, a recently reported strain from chimpanzees, and formed a distinct genogroup within the RV2 clade, supported by a bootstrap value of 85% (Figure 1). These two sequences, which branch off alone in the RV2 genogroup, independently of all other Old World monkey viral strains, may represent the prototype strains of a great apes lineage similar to that found in the RV1 genogroup. Based on the established view that herpesviruses have diverged from a common ancestor, in a manner mediating cospeciation of herpesviruses with their host species through latent infection, such findings reinforce the hypothesis of a putative RV2-related herpesvirus in humans.

The prevalence of HyloRHV2 infection, in our series of gibbons, was determined by Southern blot hybridization of heminested PCR products, with a specific oligonucleotide probe (HyloRHVpr: TTA CGG CTT TAC TGG GGT GGC GAG). Only one sample (gibbon 7) scored positive. Furthermore, we also developed a nested PCR assay using

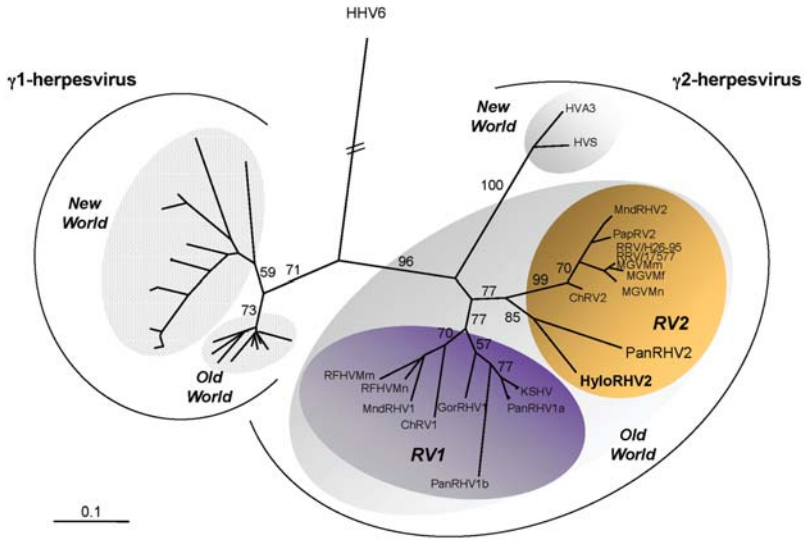

Figure 1. Neighbor-joining protein distance tree for the best 130 amino acids of DNA polymerase residues alignment. DNA sequences (primers DFASA and GDTD1B) (8) were first translated, then aligned by using ClustalX (nonphylogeneticaly informative gaps were manually removed) and analyzed by using the PROTDIST and NEIGHBOR programs in PHYLIP (available from http://evolution.genetics.washington.edu/phylip.html). Horizontal branch lengths are drawn to scale, with the bar indicating 0.1 amino acid replacements per site. Numbers in each internal branch indicate the percentage of bootstrap samples (of 1,000) in which the cluster is supported (SEQBOOT). Previously published sequences included and their accession numbers are as follows: HyloRHV2 (AY465375); PanRHV2 (AF346490); KSHV (U75698, U93872 and AF005477); PanRHV1a (AF250879 and AF250880); PanRHV1b (AF250881 and AF250882); GorRHV1 (AF250886); MndRHV1 (AF282943); MndRHV2 (AF282937 to AF282940); herpesvirus saimiri (HVS) (M31122); ateline herpesvirus 3 (HVA3) (AF083424); Chlorocebus rhadinovirus 1 (ChRV1) (AJ251573); ChRV2 (AJ251574); retroperitoneal fibromatosis–associated herpesvirus strains from *Macaca mulatta* (AF005479) and *M. nemestrina* (AF005478) called here RFHVMm and RFHVMn, respectively; *M. mulatta* rhadinovirus RRV/17577 (AF083501); rhesus monkey rhadinovirus (RRV/H26-95) (AF029302); baboon γ-herpesvirus (PapRV2) (AY270026); Macaca γ-virus strains from *M. mulatta* (MGVMm) (AF159033), *M. fascicularis* (MGVMf) (AF159032), and *M. nemestrina* (AF159031); and HHV6A (X83413). Other sequences used to construct the phylogenetic trees (especially Epstein-Barr virus and related strains) have been published mainly by Ehlers et al. (9) and are named in the legend of Figure 2.

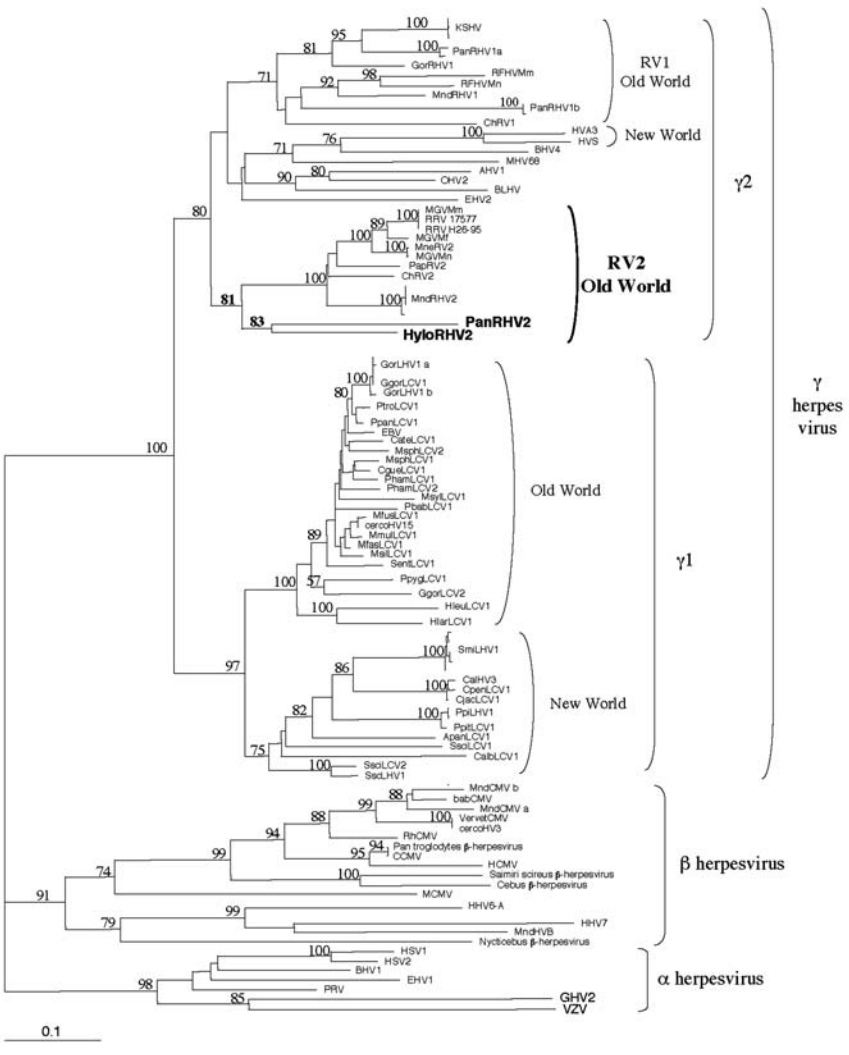

Figure 2. Phylogenetic tree resulting from analysis of selected 351-bp fragments of herpesvirus DNA polymerase gene, which is available for all viruses. The DNA sequences were first aligned by using ClustalX (nonphylogenetic informative gaps were manually removed), then the phylogeny was derived by the neighbor-joining method applied to pairwise sequence distances calculated by the Kimura two-parameter method (transition-to-transversion ratio set at 1.15 as expected by a previous Maximum Likelihood analysis). Horizontal branch lengths are drawn to scale, with the bar indicating 0.1 nucleotide replacements per site. Numbers at each node indicate the percentage of bootstrap samples (of 1,000) in which the cluster to the right is supported. Brackets on the right indicate previously defined subfamily and genus herpesviral classification. Previously published sequences and their accession numbers, as well asan Adobe Acrobat file of this phylogenetic tree, are available online at http://www.cdc.gov/ncidod/EID/vol10no5/03-0964-G2.htm

specific primers targeted to the new HyloRHV2 strain (HyloRHVs: GCA TCC CTC CCT GAC AGA GAA TG, HyloRHVas: CAT CGT GCG TCC CTG CAG CG). PCR products hybridization with the specific internal oligonucleotide probe (HyloRHVpr), only detected the same positive sample.

We also tried to specifically amplify an RV2-related virus in our series of DNA samples from orangutans using novel and specific primers targeted to RV2 genogroup strains. None of the 27 DNA scored positive, a result which might be explained either by the absence of an orangutan RV2-related virus in these DNA samples or by its presence at a level not detectable by our method, a situation similar to that observed for KSHV infection in humans. Indeed, in PBMC DNA, PCR analyses detect KSHV viral fragments in only 10% to 20% of KSHV-seropositive healthy persons (15).

In conclusion, our data demonstrated for the first time the existence of a gibbon rhadinovirus. Furthermore, after the recent demonstration of two distinct *Rhadinovirus* lineages within the common chimpanzees (7), and based on the known molecular evolution of herpesviruses, our findings of a novel RV2 virus in gibbon may suggest the possible existence of a novel γ-2-herpesvirus in humans, belonging to the RV2 genogroup.

Acknowledgments

We thank Ernst Verschoor, Klaus Eulenberger, Thierry Petit, and Jacques Rigoulet for their great help in obtaining some of the orangutan blood samples; Vincent Lacoste for critical review of this manuscript and continuous interest in this work; and Laurent Meertens for advice regarding phylogenetic studies.

Renan Duprez and Emmanuelle Boulanger are financially supported by la Ligue Nationale Contre le Cancer and l'Agence Nationale de Recherche sur le SIDA (ANRS), respectively.

Mr. Duprez is a Ph.D. candidate with primary research interests in the clinical and molecular epidemiology and phys-

iopathology of γ-herpesviruses in humans, particularly Kaposi sarcoma–associated herpesvirus clonality.

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Increase in Imported Dengue, Germany, 2001–2002

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Dengue fever is a reportable disease in Germany. Surveillance data from 2001 and 2002 were analyzed and compared to travel patterns. Imported dengue fever increased strongly in this time. Most infections were acquired in Southeast Asia, specifically Thailand. The 2002 epidemic in Brazil was also reflected in these data.

Dengue fever is endemic in many tropical regions worldwide (1,2). The disease is caused by any of four serotypes of dengue virus, a flavivirus. The World Health Organization estimates that 50 million infections and 22,000 dengue-related deaths occur annually. In many countries, the incidence of dengue fever in 2002 increased compared to previous years. The geographic range of dengue continues to expand, as a recent large outbreak in Australia's Northern Queensland demonstrates (3).

Travel to dengue-endemic areas carries the risk of acquiring the disease. Each year, an estimated 3 million German residents spend time in such countries. Country-specific risk for travel-associated dengue fever needs to be monitored to focus pretravel advice. In the absence of data on the true incidence in travelers (including asymptomatic infections and those not coming to medical attention in Germany), cases of symptomatic imported dengue fever diagnosed in Germany indicate temporal and geographic trends in all travel-associated dengue infections.

An improved surveillance system for mandatory case reporting of infectious diseases, including dengue, was implemented in Germany in January 2001. Under the Infectious Disease Control Act, German laboratories must notify local public health authorities of test results fulfilling the case definition for acute dengue virus infection, i.e., detection of viral antigen or RNA, a fourfold or greater increase in antibody titers between acute- and convalescent-phase serum samples, or detection of immunoglobulin (Ig) M antibodies to a dengue virus. The inclusion of positive IgM test results, typically the first laboratory evidence to indicate infection, enhances timeliness of reporting. In most cases, paired serum samples are tested. Local authorities then gather additional information on the patient (clinical signs, demographics, travel destination). If the case definition is fulfilled (clinical dengue plus definitive or probable laboratory evidence), the case is reported through state authorities to the central database at the Robert Koch Institute.

The Study

We analyzed surveillance data on temporal trends, demographics, and country of infection from January 2001 to December 2002. Using recent air travel data, we calculated relative country-specific dengue fever risks for travelers from Germany. Information on the numbers of air travelers from Germany to foreign destinations in 2002 is available from the Federal Statistical Office (Statistisches Bundesamt, air tourism statistics, 2002), which receives reports on ticketed destinations from all airlines that board passengers at German airports. The Thailand Authority of Tourism provides monthly statistics on the number of visitors arriving from Germany in 2002 (available from http://www.tat.or.th/stat/download.htm), as does the Brazilian Tourist Office for 2001 (available from http://www.brazil.org.uk/page.php?cid=1195). On the basis of likely month of infection, monthly risk for dengue per 100,000 travelers from Germany to Thailand and Brazil was calculated.

Sixty cases meeting the case definition were reported in 2001, and 231 cases were reported in 2002. Case reports rose continuously from 7 cases in the first quarter of 2001 to 82 cases in the second quarter of 2002 (Figure 1). In both years, 55% of cases were male. The median age at infection was 37.5 years (range 20–62 years) in 2001 and 34 years (range 5–71 years) in 2002. Travelers to Thailand ($n = 114$, median age 31.5 years) were significantly younger than travelers to Brazil ($n = 40$, median age 40 years) ($p < 0.001$). Six cases had hemorrhagic signs, but none fulfilled the World Health Organization case definition for dengue hemorrhagic fever or dengue shock syndrome. No deaths from dengue fever were reported.

Information on travel history was available for all cases in 2001 and for 98% of 2002 cases. Overall, 39.4% of infections were acquired in Thailand (2001: 36.1%, 2002: 40.6%). In 2001, stays in Venezuela (8.3%), India (6.6%), and Cambodia (6.6%)—all countries endemic for dengue fever—were also frequently implicated. The proportion of cases imported from Brazil rose from 4.9% in 2001 to 15.5% in 2002 ($p = 0.05$). In the first half of 2002, 25.6% of all cases were associated with travel to Brazil. Cases imported from Thailand peaked in the second and third quarter of 2002. Venezuela contributed 1.7% of cases in 2002, compared to 8.3% in 2001 ($p = 0.02$). A case of nosocomial dengue virus transmitted by needlestick injury was observed in a German hospital nurse in 2002 (4).

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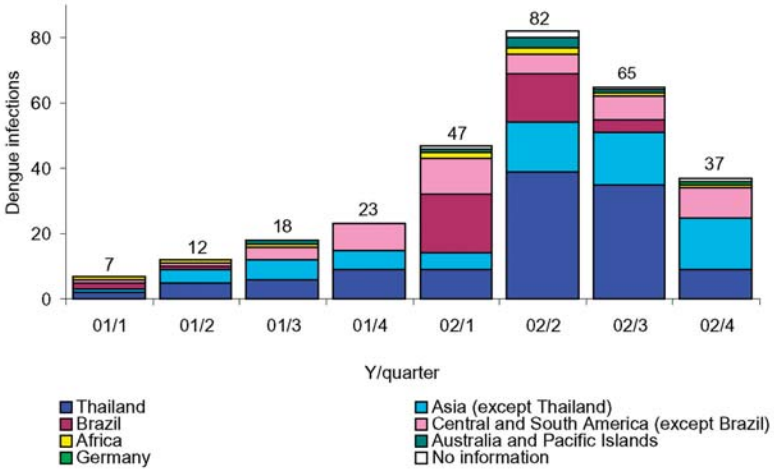

Figure 1. Cases of dengue fever reported in Germany 2001 (n = 60) and 2002 (n = 231) by region of acquisition.

Relatively high country- or area-specific incidence rates among German travelers were noted for Thailand (27.9/100,000 travelers), Brazil (22.8/100,000), South America except Brazil (21.1/100,000), the Lesser Antilles (19.1/100,000), the Central American mainland south of Mexico (16.3/100,000), the former Indochina (15.2/100,000), and Indonesia (14.8/100,000) (Table).

The incidence of dengue fever among German travelers to Thailand ranged from 2/100,000 in January and February to >70/100,000 in April (Figure 2). While travel peaks during the winter months, dengue incidence was strongly elevated during the mid-year rainy season and the month preceding it. Numbers of travelers to Brazil were slightly higher from January to March compared to the rest of the year (Figure 3). The incidence of dengue fever showed a distinct peak in February and March, reaching 39/100,000 travelers in March, and was very low from May to December.

Conclusions

Some of the steady increase in case reports in early 2001 likely reflects knowledge and acceptance of the recently improved surveillance system among physicians and laboratories who diagnose dengue. The further steep

Table. Regional risk for dengue fever in German travelers, 2002

Region	Country or area	No. of travelers flying in from Germany	Mentions of destination(s) among dengue patients	Incidence (cases/ 100,000 air travelers from Germany)
Southeast Asia	Thailand	347,569	97	27.9
South America	Brazil	162,264	37	22.8
South America	Colombia, Venezuela, Suriname, Trinidad and Tobago, Aruba, Curaçao, Bonair	61,739	13	21.1
Central America	Lesser Antilles Islands[a]	20,989	4	19.1
Central America	Guatemala, Honduras, Belize, El Salvador, Nicaragua, Costa Rica, Panama	24,564	4	16.3
Southeast Asia	Laos, Vietnam, Cambodia (Indochina)	65,963	10	15.2
Southeast Asia	Indonesia	88,053	13	14.8
Africa	Ghana	22,900	3	13.1
South Asia	India, Sri Lanka	220,169	18	8.2
South America	Ecuador, Peru, Bolivia	43,928	3	6.8
Southeast Asia	Philippines	54,231	3	5.5
Southeast Asia	Malaysia	43,698	2	4.6
Central America	Greater Antilles Islands[b]	429,614	9	2.1
Asia	Asia, Southeast Asia, Taiwan, Singapore	No data available	7	–
Australia and Pacific Islands	Australia, Pacific Islands	No data available	6	–
Africa	Africa, Cape Verde Islands, Congo	No data available	3	–
Central America	Mexico	No data available	1	–
			Total: 233[c]	

[a]Lesser Antilles: Antigua and Barbuda, Barbados, Dominica, Grenada, Guadeloupe, Martinique, St. Lucia, St. Vincent, and Grenadines.
[b]Greater Antilles: Bahamas, Cayman Islands, Dominican Republic, Haiti, Jamaica, Cuba.
[c]A total of 233 destinations were mentioned by 225 patients (6 mentioned 2 destinations, l mentioned 3); 5 patients did not provide information about predisease travel, and l case arose in Germany.

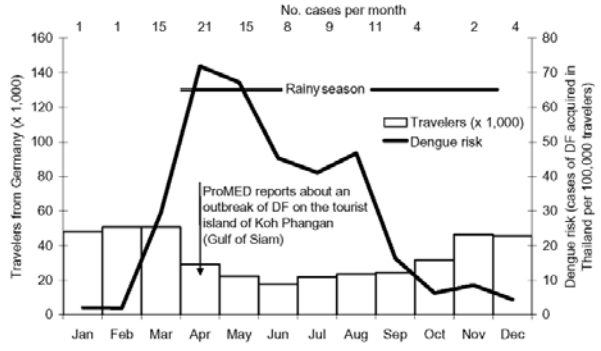

Figure 2. Risk for dengue fever (DF) among travelers to Thailand, 2002.

rise in German case reports, particularly during late 2001 and the first half of 2002, corresponds to a surge of local dengue reporting from many dengue–endemic areas and likely reflects a true increase in imported cases. By number of reports of travel-associated infectious diseases, dengue fever is second only to malaria (≈1,000 cases per year, with a 15% drop in cases from 2001 to 2002) in Germany. A parallel voluntary sentinel surveillance system for imported tropical infections in Germany recorded 78 dengue cases in 2001 and 125 cases in 2002, respectively (5). For this system, sites include infectious disease and tropical medicine centers, as well as offices of general practitioners specialized in travel medicine. By definition, there is much overlap of cases between the Robert Koch Institute's mandatory reporting surveillance system, and the voluntary sentinel system. The fact that in 2001 more cases were reported to the sentinel system compared to the institute's surveillance shows that reporting in the latter system was incomplete in its first year. However, completeness of reporting was much improved in 2002.

The spectrum of countries of infection reflects both predominating travel destinations and local trends in dengue fever endemicity, with risk for individual travelers high in both popular tourist destinations, such as Thailand or Brazil, as well as some regions visited by smaller numbers of travelers, including some Caribbean islands. Within countries endemic for the disease, dengue risk varies by place, season, and year. Urban areas can have intense and prolonged local epidemics. Introduction of a dengue virus serotype for which the population lacks immunity can cause particularly high incidence, and climate may have a strong influence on vector populations. Such fluctuations likely influenced German data.

The increase in cases from the fourth quarter of 2001 to the first quarter of 2002 is mainly due to cases imported from Brazil. During the first quarter of 2002, the state of Rio de Janeiro recorded an incidence that was 6.5 times higher than it had been in January through March of 2001. This state alone accounted for almost 50% of the total cases in Brazil during this period (6), including an urban epidemic in the city of Rio de Janeiro. Rio draws large numbers of German tourists, especially during the festival of Carnival, which most likely contributed to the high number of cases acquired in Brazil in February and March 2002 (Figure 3). In contrast, the decrease in incidence in neighboring Venezuela (from 338/100,000 in 2001 [7] to 153/100,000 in 2002 [8]) corresponded to a significant decrease in the percentage of German travelers who acquired the disease there.

The peak in cases imported to Germany in the second and third quarter of 2002 reflects the dengue season in Thailand and other parts of Southeast Asia. In Thailand, the disease is associated with the rainy season, which varies regionally but in most areas starts around April. In mid-April of 2002, an out-of-season outbreak was reported at the island resort of Koh Phangan (9), which may explain the high incidence among German travelers in March and April. Our data highlight the contribution of Southeast Asia as an area where German travelers acquire dengue fever. These findings agree with those from a Swedish case-control study, which identified travel to the Malay Peninsula as an independent risk factor for imported dengue (10) and with reports from a European network of institutions of tropical medicine (11). Although nosocomial transmission of the virus in dengue-nonendemic areas is rare, the case detected by our surveillance system clearly shows the potential of bloodborne virus transmission and the need to follow strict hygiene precautions when treating dengue patients.

The new infectious disease surveillance system in Germany, based on clinical case definitions and laboratory confirmation, is one of the few national surveillance systems in industrialized countries to include dengue fever with a specific case definition. As asymptomatic and mild infections are known to occur, some proportion of infections will escape diagnosis (12,13). Dengue is an important differential diagnosis of fever in travelers to endemic areas (12,14). In a German study, travelers who had fever after returning from dengue-endemic areas had dengue antibody

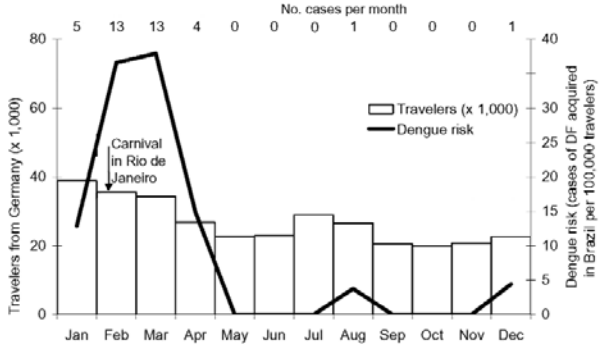

Figure 3. Risk for dengue fever (DF) among German travelers to Brazil, 2002 (Tourism data from 2001).

seroprevalence rates of 7% (15). As long distance travel expands, increasing numbers of travelers are potentially exposed to dengue viruses and more often exposed sequentially to multiple serotypes of dengue virus, increasing their potential risk for dengue hemorrhagic fever or dengue shock syndrome. Additional serologic studies in representative samples of symptomatic and asymptomatic travelers are needed to investigate the risk in defined areas.

In its second year, Germany's dengue surveillance demonstrated a rough parallel in the rate of returning travelers with dengue fever and its incidence in the places they had visited. One strength of this study was its analysis of trends on the basis of incidence specific for country of destination. Systematically collected and analyzed surveillance data on imported infections help formulate region-specific travel advice in addition to information on avoiding the vectors of dengue fever.

Acknowledgments

We thank all reporting laboratories and physicians, as well as local and state health departments, for their cooperation in collecting these surveillance data.

Dr. Frank is an infectious disease epidemiologist and geographer at the Robert Koch Institute, Berlin, Germany. Her research interests include enteric infections, viral hepatitis, zoonoses, and tropical infections.

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Corvidae Feather Pulp and West Nile Virus Detection

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We evaluated cloacal swab, vascular pulp of flight feather, and kidney and spleen pool samples from carcasses of members of the family *Corvidae* as sources of West Nile virus (WNV). The cloacal swab, kidney and spleen pool, and feather pulp were the source of WNV in 38%, 43%, and 77%, respectively, of the carcasses.

Samples from carcasses of the family *Corvidae* have been used in the surveillance for West Nile virus (WNV) since the virus was detected in the United States in 1999 (1). Various laboratories have used organs such as brain, kidney, and spleen to isolate the virus in cell culture, or to detect the virus by using a variety of techniques, or both (2). WNV surveillance efforts (3,4) have reported 50%–70% of corvids as WNV positive. Postmortem oral and cloacal swabs, along with the brain, are suitable for detecting the virus in experimentally infected birds (5). Previous studies have shown that WNV may be isolated from feather pulp of experimentally infected crows (National Wildlife Health Center, unpub. data). We describe the results of WNV isolation attempts from cloacal swabs, kidney and spleen pools, and the flight feathers containing vascular pulp (6) of dead American Crows (*Corvus brachyrhynchos*) and Blue Jays (*Cyanocitta cristata*) that were found in the field and suspected of being WNV infected.

The Study

Specimens were obtained at necropsy from a group of 28 American Crow and 56 Blue Jay carcasses. These birds were submitted, from August through October, as field cases in the course of the larger 2002 wildlife surveillance effort for WNV at the U.S. Geological Survey (USGS), National Wildlife Health Center, in Madison, Wisconsin. Birds received for WNV surveillance were evaluated for WNV but not for cause of death. Kidney and spleen pools, cloacal swab, and feather pulp were collected from each of these 84 birds received from the following nine states: Alabama, Illinois, Kansas, Maryland, Missouri, North Dakota, Pennsylvania, Texas, and Virginia. The birds in this study were obtained after WNV was initially detected in birds from each state; only those carcasses judged to be fresh were sampled.

At necropsy each bird was examined for wing flight feathers (remiges) and tail flight feathers (retrices) that contained vascular pulp. These feathers were pulled from the feather follicle and aseptically cut at the distal end of the umbilicus (6). The umbilicus, containing vascular pulp, was placed in viral transport media (7). At least one and up to three feathers were collected from American Crows and at least four and up to six were collected from Blue Jays. Also at necropsy, cloacal samples were taken with Dacron swabs that were swirled in viral transport media, squeezed out against the side of the collection tube, and then discarded. Kidney and spleen samples were aseptically collected at necropsy. Samples were kept chilled at 4°C, and any not processed within 24 hours after they were obtained were stored at –80°C.

In the laboratory, a 10% (wt/volume) kidney and spleen pool suspension was prepared in viral transport media. The kidney and spleen pool suspension was blended in a Stomacher 400 Circulator (Seward, Norfolk, UK) until it appeared homogeneous. The vascular pulp was aseptically removed from the umbilicus with forceps, and viral transport media was added to the available feather pulp mass to produce a 10% (wt/volume) suspension. The cloacal swab and feather pulp suspensions were vortexed until they appeared homogeneous. The kidney and spleen pool, cloacal swab, and feather pulp suspensions were centrifuged at 800 x *g* for 30 min at 4°C, and 1 mL of the supernatant was injected onto an established Vero (ATCC CRL-1587) cell monolayer in 12-cm^2 (culture surface area) bottles. These bottles were incubated at 37°C and 2% CO_2 and read periodically over 7 days for viral cytopathic effect (CPE). To screen for WNV, cell culture bottles showing viral CPE involving at least 75% of the Vero monolayer were harvested after one freeze-and-thaw cycle and tested for WNV by reverse transcriptase–polymerase chain reaction (RT-PCR) (8). The RT-PCR test was also used to determine whether feather pulp or cloacal swab suspensions, negative for virus isolation, contained quantities of WNV below the detection level of cell culture. The number of WNV plaque forming units (PFU) was determined (9) to evaluate the quantity of virus in various kidney and spleen pool, cloacal swab, and feather pulp samples.

With the screening method described here, WNV was isolated from 65 (77%) of 84 corvids. Of the 65 WNV-positive birds, WNV was isolated from 100% (65/65) of the feather pulp samples, 55% (36/65) of the kidney and spleen pool samples, and 49% (32/65) of the cloacal swabs. WNV was isolated from all three samples for 25% (21/84) of all birds tested. Attempts at virus isolation were significantly ($p \leq 0.001$) more successful from feather pulp than from either kidney and spleen pool or cloacal swab.

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The ability to successfully isolate WNV from either the kidney and spleen pool or the cloacal swab was essentially the same ($p \geq 0.5$).

The feather pulp or cloacal swab samples from birds from which WNV was not isolated were also negative for WNV by using RT-PCR. Comparisons of the quantity of WNV in 0.1 mL of sample suspension indicated that more PFU of WNV were in the feather pulp than cloacal swab or kidney and spleen pool suspensions (Table). A comparison of the number of PFU in the feather pulp samples and cloacal swabs from the same 12 birds showed that the feather pulp had significantly ($p \leq 0.0005$) more.

Table. Logarithmic titers of West Nile virus infectious particles (per 0.1 mL of 10% tissue suspension) present in each type of sample, as detected by plaque assay in Vero cells

Type of sample	No. tested	Median (range)
Kidney/spleen pool	7	1.0 (≤1.0 to 3.3)
Cloacal swab	12	1.9 (≤1.0 to 4.0)
Vascular pulp of flight feather	12	4.9 (3.5 to ≥7.4)

Conclusions

WNV isolation from feather pulp is a relatively sensitive assay for surveillance of corvid carcasses. Of the 84 tested, 77% (65/84) of the birds were WNV positive by feather pulp alone, 43% (36/84) were positive by kidney and spleen pool alone, and 38% (32/84) were positive by cloacal swab alone. On the basis of our determination of the WNV titer in feather pulp, cloacal swab, and kidney and spleen pool, these results could be explained by the fact that much more virus was present in the feather pulp suspension. The 23% (19/84) negative birds consisted of 16 Blue Jays and 3 American Crows, representing birds that may have died of causes other than WNV infection. Other causes may include other infectious agents, toxins, or trauma not related to concurrent WNV infection.

Previous studies of Eastern equine encephalitis virus in Ring-necked Pheasants (*Phasianus colchicus*) and avian leucosis virus in domestic chickens (*Gallus gallus*) found virus in feather pulp up to 7 days beyond detection in blood (10–12). The virus titer in feather pulp was also much greater than in blood or cloacal swab. Avian leukosis virus could be detected in feather pulp even after antibody was detected. In a recent publication (13), RT-PCR was used to detect WNV in the "skin including feather tips" of goslings experimentally infected with the virus. The authors of that publication concluded that blood and skin containing feather tips could, through cannibalism, horizontally transmit sufficient virus to directly infect contact control goslings.

The duration and timing of the molt is a limiting factor in using the feather pulp sample. In much of the United States, the American Crow molt will occur from July through September, the Blue Jay from June through October, and the Common Raven (*Corvus corax*) from May through October (14). A feather pulp sample from corvids in the United States will therefore be available during the height of the WNV season. We recommend collecting and testing the feather pulp, considering the apparent high rate of success in detecting WNV.

The feather pulp sample is nonlethal and could be taken from birds trapped live, sampled, and released. Sufficient WNV appears to be available in samples obtained from corvid carcasses suspected to be WNV infected to infect cell culture. since none of the negative cloacal swab or feather pulp samples were positive by RT-PCR. However, for subclinical infections or from other species of birds, additional testing may be necessary to determine whether the amount of virus available in feather pulp will be sufficient for detection by virus isolation or RT-PCR.

Acknowledgments

The authors thank Nathan Ramsay, Dottie Johnson, Heather Gutzman, and Michelle Oates for obtaining the postmortem specimens used in this study and Scott Wright, Carol Meteyer, Robert McLean, and Tracy McNamara for various contributions.

Funding was provided by the U.S. Geologic Survey and the Centers for Disease Control and Prevention.

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Fatal Spotted Fever Rickettsiosis, Kenya

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We report a fatal case of rickettsiosis in a woman from the United States living in Kenya, who had a history of tick exposure. Immunohistochemical staining of skin, kidney, and liver demonstrated spotted fever group rickettsiae. The clinical findings, severity, and fatal outcome are most consistent with *Rickettsia conorii* infection.

Rickettsia are gram-negative, obligate intracellular bacteria that are able to invade various eukaryotic host cells such as reticuloendothelial cells and vascular endothelial cells in vertebrates, and epithelial cells in their invertebrate arthropod vectors. Rickettsiae are transmitted to humans through infected arthropods, and hard ticks serve as both vectors and reservoir hosts for most spotted fever group (SFG) rickettsiae (1).

Two SFG rickettsiae are most commonly identified in Africa, *Rickettsia conorii* and *R. africae*. *R. conorii*, the etiologic agent of Kenyan tick typhus or more commonly known as Mediterranean spotted fever, is transmitted predominately through the bite of infected *Rhipicephalus* ticks; it has been reported in Central and South Africa (2). *R. africae*, the causative agent of African tick bite fever, is transmitted by *Amblyomma* ticks and has also been reported in South and Central Africa, including Kenya (3,4). *R. africae* has also been identified in 119 travelers who had recently visited South Africa, Swaziland, Lesotho, Gambia, Tanzania, Kenya, Gabon, and Côte d' Ivoire (5). A third SFG rickettsia, *R. aeschlimannii*, has recently been described in South Africa (6).

We describe a fatal case of spotted fever rickettsiosis in a missionary from the United States who lived in a rural town in the central district of Kenya, approximately 70 km north of Nairobi. Although the patient received prompt medical attention at a private hospital, her illness was initially diagnosed as malaria, and this misdiagnosis possibly contributed to the fatal outcome. This case emphasizes the need to consider spotted fever rickettsioses in the diagnosis of patients with fever in Africa, and the importance of determining the distribution and prevalence of these diseases in this region.

Case Report

During February 1999, a 39-year-old woman sought treatment at a hospital in Kijabe, Kenya, for a "boil" on her lower left leg. Over the next 3 days, headache, myalgia, chills, sweats, and a temperature of 38.5°C developed. The patient was a missionary from the United States, who frequently hiked in the forest around Kijabe with her dog. Four days before discovering the lesion on her leg, she had taken her ill dog to the local veterinarian. The dog, which was frequently infested with ticks, was diagnosed with "tick-borne fever" (an unspecified illness in dogs, associated with tick infestation) and was treated with imizol (imidocarb dipropionate). The dog's symptoms resolved within 3 days.

Five days after the onset of her symptoms, the patient returned to the hospital when her condition had not improved. Hematologic evaluation showed a marginal decrease in leukocytes to 4,200/mm^3 (80% polymorphonuclear cells, 2% bands, 17% lymphocytes, and 1% eosinophils), and thrombocytopenia (107,000/mm^3). No malaria parasites were detected on thick or thin blood smears. The patient was treated with acetaminophen and released from the hospital.

The next day, the patient returned to the hospital after the onset of vomiting and a nonpuritic, blotchy, macular rash on her arms and abdomen. Because she had traveled recently to a malaria-endemic area, malaria was diagnosed. Treatment was initiated with three tablets of Fansidar (500 mg sulfadoxine and 25 mg pyrimethamine per tablet). The patient reported some improvement of symptoms that evening, and by the following day, she ceased vomiting and her fever was reduced.

On the day 9 of her illness, a generalized macular rash covered her full body, sparing her palms and soles. Her pharynx was red. Her abdomen was tender in both upper quadrants. A viral exanthem as well as malaria responding to treatment were diagnosed. Vomiting and fever began again that afternoon. That evening she became mildly confused and was admitted to Kijabe African Inland Church Mission Hospital. Her temperature was 38°C; she was tachypnic and had a nonproductive cough. She had mild epigastric tenderness, but spleen and liver margins were not palpable. An eschar surrounded by a 1.5 cm purpuric border was identified on her lower left leg. Fansidar-resistant malaria as well as a viral exanthem were diagnosed, and she was treated with intravenous fluids, quinine, acetaminophen, and promethazine. The following day, her

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temperature was 39.9°C, and she was unable to follow verbal commands. Multiple seizures developed, and she went into cardiopulmonary arrest. Despite attempts at cardiopulmonary resuscitation, the patient died.

Histopathologic findings included extensive epidermal and dermal necrosis and acute inflammation at the site of the eschar with focal, transmural, mixed inflammatory cell infiltrates involving dermal blood vessels (Figure 1). The liver showed portal triaditis and focal eyrthophagocytosis by Kupffer cells. The spleen showed multiple microscopic necrotic foci involving red and white pulp. Multifocal lymphohistiocytic infiltrates were identified in the interstitium of the heart and kidneys. The lungs showed mild pulmonary edema and congestion.

Formalin-fixed, paraffin-embedded tissue and serum specimens were sent to the Centers for Disease Control and Prevention for evaluation. Immunohistochemical (IHC) staining for SFG *Rickettsia* demonstrated rickettsiae and rickettsial antigens in sections of heart, spleen, and kidney localized within and around vascular endothelial cells and reticuloendothelial cells, predominantly in areas associated with perivascular mononuclear inflammatory infiltrates (Figure 2).

A single serum sample, collected on the ninth day of illness, demonstrated neither immunoglobulin (Ig) G nor IgM antibodies reactive with *R. conorii* when tested by an indirect immunofluorescence antibody assay at a screening dilution of 1:16. To further define the causative agent, we attempted to amplify a portion of the rickettsial *rOmpA* gene, as we have done for ticks from this region (4), using DNA extracted from the paraffin-embedded sections of heart, spleen, and kidneys. Briefly, paraffin-embedded tissue samples were deparaffinized in xylene, and rinsed in absolute ethanol. Genomic DNA was extracted by using the Wizard genomic DNA purification kit (Promega, Madison, WI), according to the manufacturer's protocol. Amplification of a 635-bp fragment of the *rompA* gene encoding the SFG-specific 190-kDa protein was attempted by using primers Rr190.70p (7) and Rr190.701 (8). Genomic DNA isolated from *R. montanensis*–infected Vero cells, and water served as positive and negative controls, respectively, for the polymerase chain reaction (PCR).

Conclusions

Immunostaining demonstrated an SFG rickettsial infection as the cause of the patient's death; yet, because the IHC assay reacts with several species of SFG rickettsiae (9), a specific etiologic agent was not determined. Serologic evaluation of a serum specimen obtained late in the course of this patient's illness failed to demonstrate antibody reactive with SFG rickettsiae; however, some patients with fatal rickettsial infections die before notable levels of these antibodies are detected (9).

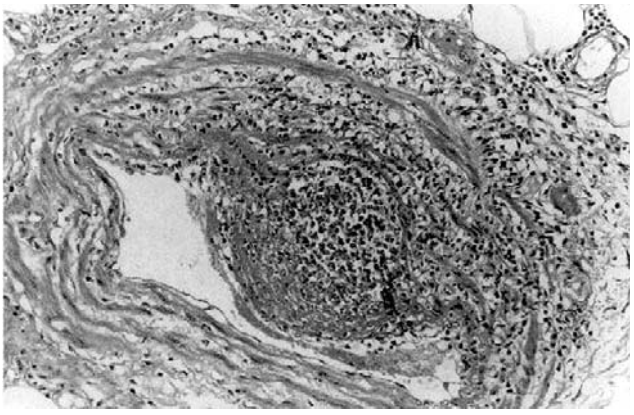

Figure 1. Vasculitis and edema involving medium-sized artery in the subcutaneous fat at the site of the eschar (hematoxylin and eosin stain; original magnification x100).

Additionally, repeated attempts to amplify rickettsial DNA from numerous thin-section preparations from paraffin-embedded tissues were unsuccessful. Therefore, we were unable to characterize the infecting rickettsiae using molecular techniques. Sample fixation procedures may decrease the sensitivity of the PCR, as seen in diagnostic assays for other microorganisms (10). Further work is needed to determine the utility of PCR as a reliable method of detecting rickettsial DNA in paraffin-embedded tissues.

The spectrum of rickettsial infections in Africa ranges from mild to severe. *R. conorii* causes a moderate to severe illness with a case-fatality rate of approximately 3%. Disease is most often characterized by a single eschar and a generalized maculopapular rash that may involve the palms and the soles (11). By contrast, *R. africae* causes a generally milder, self-limiting disease with fever, multiple eschars, regional lymphadenopathy, and rash in approximately 16% to 46% of patients (3,12).

Anecdotal reports and some animal and human studies suggest that sulfa-containing antimicrobial agents exacerbate the clinical severity of rickettsial infections (13,14), and the patient described here received a sulfa-containing antibiotic after malaria was diagnosed. However, an early correct diagnosis and prompt administration of effective antirickettsial therapy (e.g., doxycycline or another tertacycline) remain the primary determinants of successful clinical outcomes for patients with spotted fever rickettsioses. Evidence is accumulating that tick-borne rickettsioses are an underreported and underappreciated cause of illness in sub-Saharan Africa. For example, while investigating this case we discovered that a mild febrile illness, accompanied by rash, developed in nearly one third of a group of boarding school students from the United States, who had camped at Masai Mara, a popular tourist destination; all had histories of tick bite (J. Rutherford, unpub. data). Additionally, recent identification of SFG rickettsiae

in ticks from this area confirmed the presence of *R. africae* in *Amblyomma variegatum* collected from domesticated livestock (4). Rickettsial infections in Central and sub-Saharan Africa have been reported among tourists and travelers who visited game reserves (12). The prevalence of spotted fever in local populations may be obscured by the lack of obvious rash and the overwhelming number of cases of malaria.

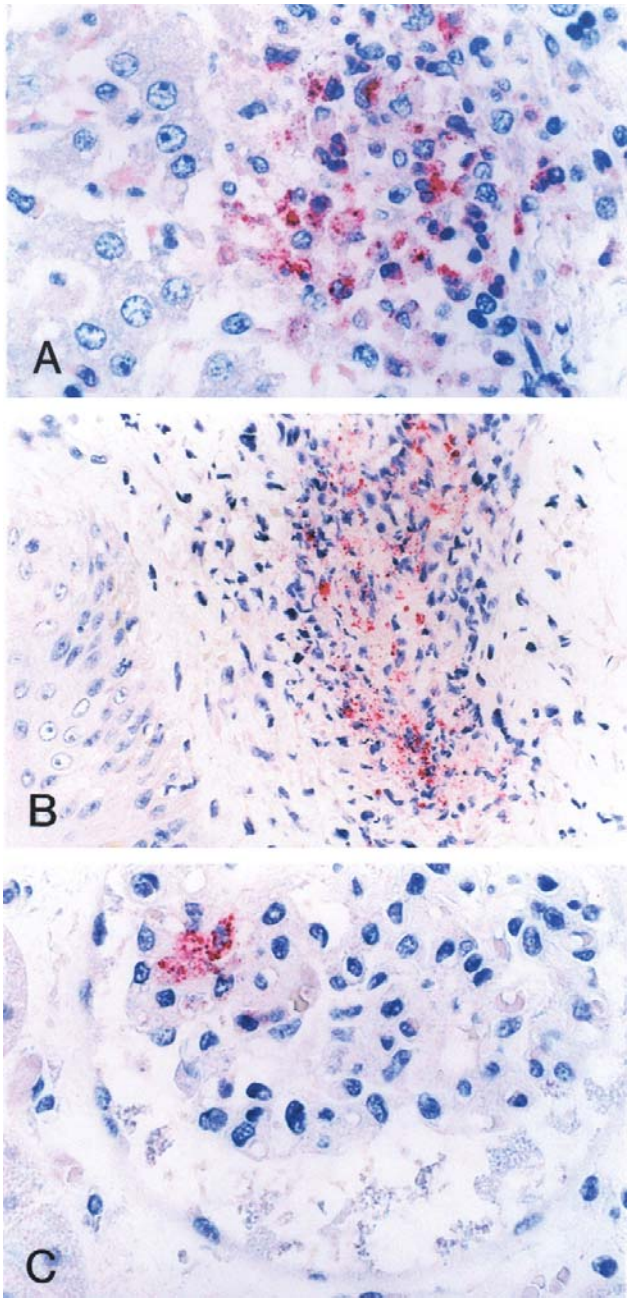

Figure 2. Immunohistochemical localization of spotted fever group rickettsial antigens in various tissues of a patient with fatal spotted fever rickettsiosis, by immunoalkaline phosphatase stain with naphthol phosphate–fast red substrate and hemotoxylin counterstain. Rickettsiae and rickettsial antigens (red) in Kupffer cells in liver (A), perivascular infiltrates in skin (B), and glomerular endothelium in kidney (C) (naphthol–fast red stain with hematoxylin counterstain; original magnifications x158).

This case report highlights both an immediate and a long-term need for rickettsial surveillance and dissemination of information about rickettsioses to health care workers in Africa. In a similar manner, physicians in industrialized countries who care for ill travelers who have visited Africa should consider rickettsial infections among the differential diagnoses of febrile disease, particularly in clinical situations in which malaria has been reasonably excluded as a cause of the patient's illness. In these contexts, further studies of the epidemiology and ecology of African SFG rickettsiae and rickettsioses are warranted.

Acknowledgments

We thank Tim Fader and Asa Barnes for their assistance in this investigation and Nordin Zeidner for review of this manuscript.

Funding for this project was provided by the United States Army Medical Research Unit-Kenya and National Institutes of Health.

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Domestic Poultry and SARS Coronavirus, Southern China

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SARS coronavirus injected intratracheally into chickens, turkeys, geese, ducks, and quail, or into the allantoic sac of their embryonating eggs, failed to cause disease or replicate. This finding suggests that domestic poultry were unlikely to have been the reservoir, or associated with dissemination, of SARS coronavirus in the animal markets of southern China.

An outbreak of severe acute respiratory syndrome (SARS) occurred in Guangdong Province, People's Republic of China, in November 2002 and spread to patients in 30 countries in Africa, Asia, Australia, Europe, and North and South America (1,2). As of July 11, 2003, SARS had been diagnosed in 8,437 patients; 813 died (1). A novel coronavirus was isolated in tissue culture or detected by reverse transcription–polymerase chain reaction (RT-PCR) from multiple respiratory specimens in many patients with SARS (2–4). The SARS-coronavirus (SARS-CoV) is proposed to be the cause of this syndrome on the basis of its association with human clinical cases (3,4) and reproduction of pulmonary lesions in experimentally challenged cynomolgus macaque monkeys (*Macaca fascicularis*) (5). Furthermore, some of the first persons identified with SARS-CoV infections were vendors in animal markets of southern China, which suggests a possible animal source (6). SARS-CoV has been detected by real-time RT-PCR or isolated from two wild mammalian species, Himalayan palm civet (*Paguma larvata*) and raccoon dog (*Nytereutes procyonoides*), in a market in southern China (7), but other studies in southern China involving six provinces and Beijing, as well as sampling of 54 wild and 11 domestic animal species, did not find SARS-CoV (8). The original source of this virus remains unknown (3). The susceptibility of different animal species within the animal meat markets is unknown.

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Coronaviruses have been identified in numerous mammalian and avian hosts. Most widely studied and of common occurrence are coronaviruses reported in chickens (infectious bronchitis virus), turkeys (turkey enteric coronaviruses), cats (feline infectious peritonitis virus and feline enteric coronavirus), dogs (canine enteric coronaviruses), swine (porcine hemagglutinating encephalomyelitis virus, porcine transmissible gastroenteritis virus, and porcine respiratory coronavirus), cattle (bovine enteric and respiratory coronaviruses), mice (Murine hepatitis virus), rats (sialodacyradenitis virus), rabbits (rabbit coronavirus), and humans (respiratory and enteric coronaviruses) (9). However, on the basis of sequence data, SARS-CoV is sufficiently different from these known group 1, 2, and 3 animal and human coronaviruses to be classified as a new group, group 4 coronaviruses (10). Most likely SARS-CoV originated from an unknown animal reservoir, not from a benign coronavirus in the human population (10,11).

Domesticated poultry species are major commodities traded in the animal markets of southern China. Poultry have been shown to be reservoirs for H5N1 and H9N2 avian influenza viruses that have crossed over and caused infections in humans from 1997 to 2003, some with fatal outcomes (12–14). Therefore, poultry should be examined as potential hosts for infection and amplification of SARS-CoV to determine any potential role they may have played during the emergence of human infections in southern China.

Groups of nine 3-week-old domestic geese (*Anser anser domesticus*), 3-week-old domestic Pekin ducks (*Anas platyrhyncos*), 4-week-old chickens (*Gallus gallus domesticus*), 3-week-old turkeys (*Meleagris gallopavo*), and 5-week-old Japanese quail (*Coturnix coturnix japonicus*) were each injected intratracheally with $10^{6.2}$ mean tissue culture infective doses ($TCID_{50}$) of Vero E6 propagated Urbani SARS-CoV per bird in a volume of 0.1 mL. The inoculum was the third passage in Vero E6 cells from the original throat swab specimen of the patient. The chickens were specific pathogen–free from an inhouse flock. The other four species were conventional birds obtained at 1 day (geese, turkeys, and ducks) or 5 weeks of age (quail) from commercial hatcheries and raised on site. Oropharyngeal and cloacal swabs were obtained on days 0, 1, 2, 3, 4, and 10 after injection from five birds per group for virus detection by real-time RT-PCR and virus isolation on Vero E6 cells. RNA for RRT-PCR was extracted with the Trizol LS reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. Two hydrolysis probe type real-time RT-PCR assays, both targeting the ORF 1b gene, were optimized and run on a Smart Cycler (Cepheid, Sunnyvale, CA) with the superscript platinum taq one-step RT-PCR kit (Invitrogen, Carlsbad, CA). Real-time RT-PCR tests included negative (noninfected tissue

culture media, infectious bronchitis coronavirus, and turkey enteric coronaviruses) and positive (Vero E6 propagated SARS-CoV) controls. Two injected birds of each species were euthanized. After necropsy, their tissues were collected for histopathologic examination (all tissue types) and virus detection (plasma, trachea, lung, spleen, kidney, and heart) on days 2 and 4 after injection, and at termination on day 10 after injection. For determination of infection, serum was collected on days 0 and 10 after injection from all birds and tested by indirect enzyme-linked immunosorbent assay for anti-SARS-CoV antibodies. Antigen used to coat plates was tissue culture propagated Urbani strain of SARS-CoV inactivated by γ irradiation (3). Secondary "anti-bird" antibody (Bethyl Laboratories, Montgomery, TX) for testing quail and goose serum or plasma, and secondary anti-duck, anti-chicken, and anti-turkey antibodies (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) for testing duck, chicken, and turkey serum and plasma, respectively, were used. Two birds of each species received uninoculated tissue culture fluid and served as the sham-inoculated groups for real-time RT-PCR, standard RT-PCR, virus isolation, and histopathologic and serologic assays.

To determine if SARS-CoV could grow in avian embryos, 9-day-old chicken eggs and 13-day-old turkey embryonating eggs were inoculated by allantoic sac route and 17-day embryonating turkey eggs were inoculated by yolk sac route; all were tested by virus isolation and real-time RT-PCR for SARS-CoV. All laboratory procedures and animal studies were conducted in biosafety level 3 agriculture (BSL-3AG) (15) facility with HEPA respiratory protection and barrier clothing procedures for personnel. General care was provided in accordance with the Institutional Animal Care and Use Committee.

To establish the comparative sensitivity of virus isolation and real-time RT-PCR tests, serial dilutions of SARS-CoV propagated in Vero E6 cell culture were tested for virus reisolation in Vero E6 cells and detection of replicase ORF 1b gene by real-time RT-PCR (16). Virus isolation was slightly more sensitive, detecting virus in two of three replicates at the 10^{-7} dilution; the real-time RT-PCR test detected SARS-CoV in three of three replicates at 10^{-5} to 10^{-6} dilution, depending on primer sets. The real-time RT-PCR assay detected virus in oropharyngeal swab specimens from two chickens on day 1 PI (Figure). Real-time RT-PCR results were confirmed by standard RT-PCR targeting the same gene (primers: SARS clone 1b For 5′-TgACAgAgCCATgCCT-3′, SARS clone 1b Rev 5'CAACggCATCATCAgA-3′) and sequencing of the amplified product. No infectious virus was isolated from any of the birds at any time from oropharyngeal or cloacal swab specimens, plasma, or tissues. Histologic examination did not identify any specific lesions. No anti–SARS-CoV–specific antibodies were detected in birds at 0 or 10 days after injection. Levels of SARS-CoV were detected corresponding to the inoculated titers in chicken and turkey embryonating eggs by real-time RT-PCR, but not by virus isolation.

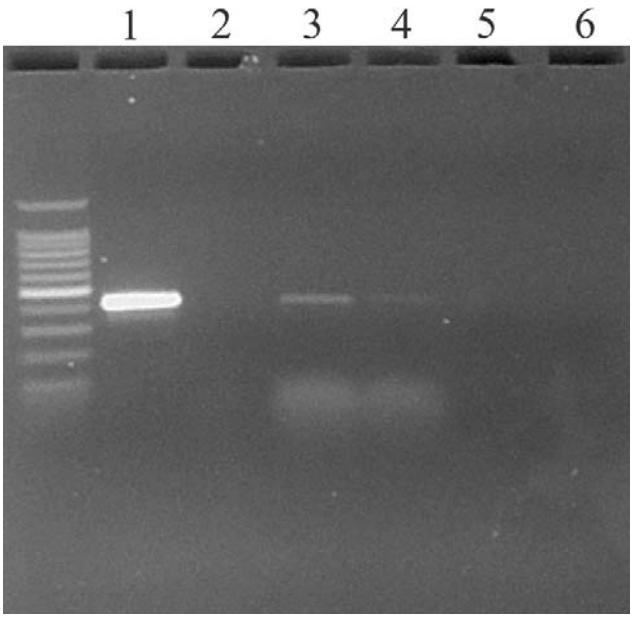

Figure. Ethidium bromide–stained agarose gel of ORF 1b standard reverse transcription–polymerase chain reaction products from oropharyngeal swabs of two chickens day 1 after injection. Lanes: 1) Positive control (severe acute respiratory syndrome coronavirus from Vero E6 culture); 2) negative control (water); 3) and 4) oropharyngeal swabs from chickens 337 and 341 at 1 day after injection; 5) cloacal swab from turkey at day 2 after injection; and 6) negative control from cloacal swab of turkey day 0 after injection.

These findings suggest that poultry were unlikely to have been infected during the recent SARS-CoV outbreak and were unlikely to have played any role as amplifiers in the animal markets of southern China. The low level of virus detected by real-time RT-PCR from the chickens and the failure to isolate virus from embryonating chicken and turkey eggs suggest that the detected virus was residual inoculum or nonviable virus and that substantial virus replication in the poultry was unlikely. In addition, this SARS-CoV was of low tissue culture passage, i.e., third passage in Vero E6 cell, which minimized the potential for increased cell culture adaptation and concomitant decrease in vivo replication. Using the original or second tissue culture passage would unlikely have resulted in substantial replication in poultry. However, the virus used in these experiments, the Urbani SARS-CoV, had a 29-nt deletion in the genome. Whether the GZ01 human virus or those from civet cats and raccoon dog containing the extra 29 nt would infect and amplify in poultry would be of interest for future research.

Acknowledgments

We thank Suzanne DeBlois and Scott Lee for excellent technical assistance.

Funding for this study was provided by the U.S. Department of Agriculture, Agricultural Research Service CRIS project #6612-32000-039.

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Mass Smallpox Vaccination and Cardiac Deaths, New York City, 1947

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In April 1947, during a smallpox outbreak in New York City (NYC), >6 million people were vaccinated. To determine whether vaccination increased cardiac death, we reviewed NYC death certificates for comparable periods in 1946, 1947, and 1948 (N = 81,529) and calculated adjusted relative death rates for the postvaccination period. No increases in cardiac deaths were observed.

Smallpox was successfully eradicated in 1980 after a global vaccination campaign by the World Health Organization. After the terrorist events of September and October 2001, the U.S. government initiated a campaign to immunize the American military and civilian first-responders in the event of an intentional release of the smallpox virus (1). From December through April 2003, smallpox vaccine was administered to 29,584 civilians and 365,000 military personnel nationwide (2,3). By March 28, four nonfatal and three fatal myocardial infarctions (MIs) had been reported. Whether these ischemic deaths were vaccine-associated or co-incidental is unclear.

To ascertain whether cardiac deaths increased after a large 1947 smallpox vaccination campaign in New York City (NYC), we examined death certificates from a 4-month period in 1947 as well as from comparable periods in 1946 and 1948. Key findings were published in an earlier article (4). We provide full results and additional methodologic detail here.

The Study

From April 4 through May 2, 1947, 6.35 million New Yorkers were vaccinated with the NYC Board of Health vaccinia strain (5). We used newspaper accounts and NYC Department of Health records to estimate the number of adults vaccinated on each of the 29 days (5). Since all of the 2003 cardiac events occurred from 4 to 17 days after vaccination, the 1947 vaccination numbers were divided equally across the same 14-day period to calculate the person-time at risk for potential cardiac death. On the basis of these estimates, we identified the 2- and 4-week "peak" risk periods in 1947.

We obtained all death certificates issued in NYC for the 4-month period between March and June, 1946–1948, from the NYC Municipal Archive. Cause of death was coded according to the International Classification of Diseases, 5th Revision (ICD-5) (6). We abstracted the date of death, age of decedent, and ICD-5–coded primary and other cause of death into an electronic database. We defined cause of death as "cardiac" if the ICD-5 codes for either cause included pericarditis (090), acute endocarditis (091), chronic endocarditis (092), myocardial disease (093), coronary artery diseases (094), and other disease of the heart (095).

We compared daily death rates during the postvaccination risk periods with rates at other times during the study period. We used Poisson regression, a generalized linear model appropriate for analysis of discrete data, to model counts of cardiac deaths (7). Counts were used instead of rates, as NYC's population remained relatively constant during the study's 3-year timeframe. We also adjusted for temporal trends in the data: a long-term trend from 1946 to 1948 (defined by weeks since January 1, 1946) and a seasonal trend between March and June (defined by days since March 1 for any given year). Secular trends were modeled with linear and quadratic terms. The main model included all cardiac deaths as the outcome variable and a dichotomous "exposure" variable indicating whether the death occurred during the 2-week risk period. Additional models examined subsets of cardiac disease and all-cause death as outcomes, as well as adjusting for noncardiac death volume.

An a priori power analysis found that the model had >90% power to detect a 5% increase in cardiac fatalities in the at-risk period. While this power would be more than sufficient to detect an excess of 2 deaths in 29,584 civilians (approximately 400 deaths in the 1947 NYC population of 6,000,000), it would not be able to detect very small elevations in risk.

At the height of the 1947 vaccination campaign, from April 17 to April 21, 500,000 to 1 million people were vaccinated daily (Figure 1). The 2-week at-risk period in 1947 was estimated to be April 22 to May 5, which encompassed 84% of the projected at-risk person-time for adverse cardiac complications. The 4-week period was

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[1]At the time of this study, Dr. Karpati was jointly affiliated with the New York City Department of Health and Mental Hygiene and the Centers for Disease Control and Prevention.

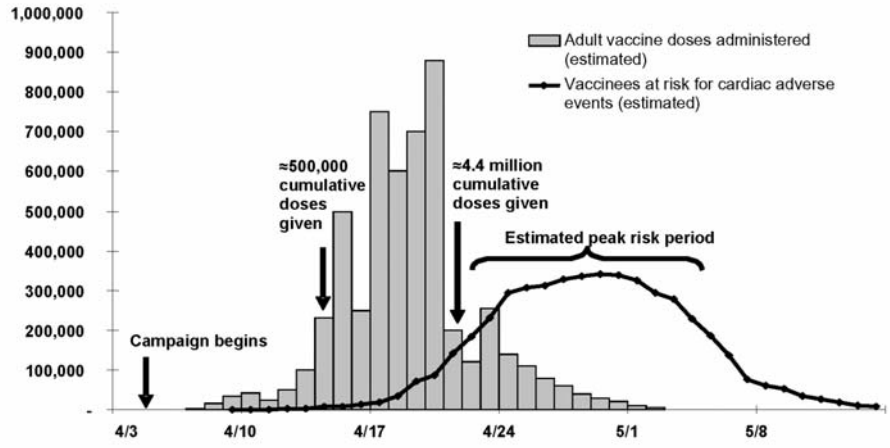

Figure 1. Adult vaccination doses administered and estimated person-time at risk for fatal cardiac adverse effects, New York City, 1947.

identified as April 16 to May 13 and included 99% of the at-risk person-time.

During the months under review in 1946–1948, 81,529 death certificates were recorded, including 519 (0.6%) records with an illegible cause of death. Of the remaining 81,010 records, 48% had heart disease listed as a cause of death. A total of 9,112 (11%) specifically referred to coronary artery or atherosclerotic disease. The number of daily deaths from heart disease in the months of March to June of 1946, 1947, and 1948 ranged from 72 to 149, with an increasing long-term trend and decreasing seasonal trend (Figure 2). In the 2-week estimated risk period in 1947, 1,545 cardiac deaths occurred of 3,156 total deaths (average 110 deaths per day, range 91–119 deaths) (Table 1).

In the main regression model (Table 2), no independent association was found between cardiac deaths and the 2-week estimated risk period. The findings remained nonsignificant when the model was restricted to those 50 to 64 years of age and when adjustments for noncardiac deaths were made (rate ratio 1.01; 95% confidence interval 0.95 to 1.06). Additional analyses examining different outcomes (all deaths, atherosclerotic deaths, or deaths due to myopericarditis) did not show any significant increase in deaths, nor did expanding the estimated risk period to 4 weeks.

Conclusions

Our analysis found no significant increase in reported cardiac deaths after the 1947 mass smallpox vaccination campaign in NYC. The campaign was unique in terms of the number of people vaccinated in one area in a short period. The high intensity and coverage of the vaccination campaign permitted a focused cardiac death assessment.

Recent reports of cardiac deaths after smallpox vaccination have raised concerns regarding the safety of the current vaccination initiative. The NYC Board of Health vaccinia strain used today is the same as was used in 1947 (5). As described in our prior publication (4), vaccinia is a DNA virus with limited antigenic variability (8), and antigenic shifts are unlikely. Regarding the vaccinated population, major risk factors, such as smoking and hypertension, were more widespread in 1947 than they are at present

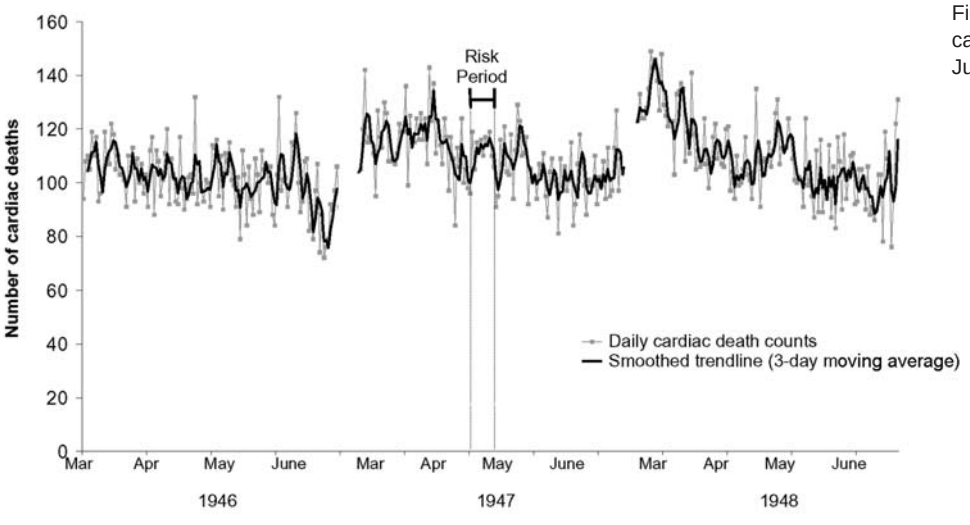

Figure 2. Daily deaths from cardiac causes, New York City, March to June, 1946–1948.

Table 1. Death counts by cause of death and postvaccination exposure period

Timeframe	All deaths	Cardiac deaths	Atherosclerotic deaths
Mar–Jun 1946	26,256	12,340	2,861
Mar–Jun 1947	27,484	13,352	3,075
Mar–Jun 1948	27,774	13,458	3,175
2-week exposure period	3,156	1,545	280

(9–11), and the death rate due to heart disease was nearly three times higher (11). If, as the 2003 cardiac fatalities suggest, cardiac risk factors increase vaccine-associated death rates, we should have seen an even-greater cardiac mortality risk in 1947.

Our analysis has some limitations. First, this analysis was ecologic, and we had no information on the vaccination status of decedents. More than 80% of the NYC population was vaccinated within the 4-week period, however, which minimizes the risk of faulty ecologic inference. The campaign urged all New Yorkers to get vaccinated, irrespective of age, health, or pregnancy (5), and the likelihood of systematic bias that would mask an association is small.

Second, death certificate information may have been incomplete or inaccurate. We extracted ICD codes for >99% of hardcopy death certificates, and missed codes were unlikely to affect the findings of the study. We also have no reason to believe that cardiac deaths were systematically misclassified in the peak risk exposure period as compared with other times. ICD-5 heart disease codes and later ICD revisions have been assessed to have a high comparability ratio, from 0.98 to 1.01 (6).

Third, assumptions pertaining to a Poisson distribution may not be appropriate for these cardiac death data (11). However, no biologically plausible concern existed for underdispersion, and goodness-of-fit statistics suggested adequate fit. Null findings of the study reduce concern for overdispersion, which could have otherwise potentially caused us to report an association that was not causal.

Finally, although the survey had substantial statistical power to detect small increases in cardiac deaths in the estimated at-risk period, extremely small increases may not have been detectable. In a large population, even small elevations in risk will produce a sizable absolute number of deaths. In light of this limitation, common to all observational studies, these findings should be interpreted in the context of other study findings.

Any one study will not likely be able to definitively rule out a causal relationship between cardiac deaths among recent vaccinees and the vaccine itself, but findings from our study provide some reassurance that the current smallpox vaccination program is unlikely to increase risk for death from coronary disease. In 1947, the commissioner of health of NYC reminded his peers, "Whenever a large-scale vaccination program is undertaken, there is always the possibility that there may be some unfortunate complications…. In New York City, there are thousands of people who become ill, and about two hundred of them die every day. Since practically every person in New York City had a recent vaccination, it was inevitable that some of them would become ill and would die. Vaccination does not stop the normal course of events. Neither should vaccination be blamed for a death from cerebral hemorrhage, nephritis, or coronary occlusion (5)."

Acknowledgments

We thank the many staff members at the New York City Department of Health and Mental Hygiene (NYC DOMH) who worked long hours to rapidly enter these data, as well as the staff of the NYC DOHMH Public Health Library (particularly Kristine Alpi, and Tamantha Smith), MIS (Julian Yuen, Anita Ho, Ron Dylan), Vital Records (Richard Genevese), Denis Nash, and the Smallpox Vaccination and Adverse Events Team. We also thank Starros Memtsoudis; Martin Kulldorf for his advice and assistance with statistical matters; Robert Chen, Susan Chu, Michael Lane, John Livengood, Linda Neff, Walter Orenstein, Richard Schreiber, Donna Stroup, Stephanie Zaza, and the Cardiac Investigation Task Force of the National immunization Program for their comments and technical assistance; and Barbara S. Chang for suggesting the analysis.

Table 2. Rate ratio (RR) and 95% confidence interval (CI) of cardiac death rates comparing postvaccination to reference periods,[a] New York City, March–June, 1946–1948

Outcome	Postvaccination period	RR (95% CI)
All cardiac deaths (ICD-5 090–095)	April 22–May 5	1.01 (0.96 to 1.07)
50- to 64-year-olds only		1.05 (0.95 to 1.15)
Atherosclerotic cardiac deaths (ICD-5 094)	April 22–May 5	1.06 (0.97 to 1.16)
50- to 64-year-olds only		1.00 (0.86 to 1.15)
Myopericarditis deaths (ICD-5 090, 093)	April 22–May 5	1.00 (0.94 to 1.07)
All deaths	April 22–May 5	1.00 (0.97 to 1.04)
All cardiac deaths (ICD-5 090–095)	April 16–May 13	0.99 (0.95 to 1.04)

[a]All models are adjusted for long-term temporal and seasonal trends.

Dr. Thorpe is a CDC epidemiologist assigned to the New York City Department of Health and Mental Hygiene. Division of Epidemiology. She is currently prioritizing research related to heart disease, diabetes, cancer prevention and control, obesity, and school health.

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Mycobacterium africanum Cases, California

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Five *Mycobacterium tuberculosis* complex isolates in California were identified as *M. africanum* by spoligotyping, single nucleotide polymorphisms, a deletion mutation, and phenotypic traits, confirming it as a cause of tuberculosis in the United States. Three of the five patients from whom *M. africanum* was isolated had lived in Africa.

Mycobacterium africanum is a member of the *M. tuberculosis* complex, which has been isolated from humans in equatorial Africa. The disease produced by *M. africanum* is similar to that caused by *M. tuberculosis* or *M. bovis*, and like *M. tuberculosis*, this organism is likely spread by aerosol transmission (1). Human tuberculosis caused by *M. africanum* has been reported in Europe (2,3). However, we are unaware of previous reports of disease caused by *M. africanum* in the United States.

The Study

M. africanum may be identified by spoligotyping (4), by specific deletion mutations (5), DNA fingerprinting by IS*6110* restriction fragment length polymorphisms (RFLP) (4), or a combination of these methods. Isolates were initially identified as *M. tuberculosis* complex by using the AccuProbe system (Gen-Probe; San Diego, CA). The isolates then underwent IS*6110*-based RFLP fingerprinting. The RFLP analyses were performed according to the method of van Embden et al. (6). In addition to providing genotyping results, RFLP fingerprinting confirmed the identification obtained with Accuprobe.

All strain typing was performed in-house at the Microbial Diseases Laboratory, California Department of Health Services. This laboratory has compiled a database (Genomic Solutions BioImage) of approximately 7,000 DNA fingerprints, typed by IS*6110* RFLP (6,7) from throughout California; most are from the San Francisco Bay area. Three isolates (from patients A, B, and C) were initially suspected of being *M. africanum* because of an epidemiologic association with Africa. These isolates were fingerprinted by IS*6110* RFLP and by spoligotyping (8). All three were found to have the "signature" spoligotype described by Viana-Niero et al. as being characteristic of *M. africanum* (4), namely, they were missing spacers 8, 9, and 39 but had spacers 40–43. Using BioImage software, we searched the laboratory's database for IS*6110* fingerprints that matched those of the three cases with an African connection. This search yielded an additional two matches, cases D and E. Isolates from cases D and E were then genotyped by using spoligotyping and found to have the *M. africanum* signature spoligotype.

The five *M. africanum* isolates were further characterized by performing standard biochemical identification tests and testing for susceptibility to pyrazinamide (PZA). Niacin production and nitrate reduction were detected as described by Kent and Kubica (9). Susceptibility to PZA was determined by using the BACTEC radiometric assay performed according to the method of Salfinger et al. (10). The *M. africanum* isolates were then examined to determine whether they had the RD9 deletion and specific *oxy*R and *kat*G sequence mutations.

Brosch et al. had reported that isolates of *M. tuberculosis* do not have the RD9 deletion, whereas other members of the *M. tuberculosis* complex, including *M. bovis*, *M. microti*, and *M. africanum* have this deletion (5). Two sets of primers for detecting the RD9 deletion, designed as described by Brosch et al., were obtained from Alex Pym at Stanford University. A ≈480-bp region was amplified by polymerase chain reaction (PCR) from *M. africanum* by using the flanking primers, and a ≈375-bp region was amplified from *M. tuberculosis* by using the internal primers. After an initial denaturation step of 10 min at 95°C, amplification was performed for 35 cycles at 95°C for 30 s, 55°C for 1 min, and 72°C for 4 min, and a final step of elongation at 72°C for 10 min. The final concentration of each component in the 50-µL PCR reaction tube was 1x PCR buffer, 2.5 mmol/L of $MgCl_2$, and 1.5 U of AmpliTaq Gold polymerase using AmpliTaq Gold kit with GeneAmp 10x PCR buffer II and $MgCl_2$ solution (Applied Biosystems, Foster City, CA), 1.25 mmol/L of deoxynucleotide triphosphate mix (Applied Biosystems), 0.2 µmol/L of each primer, 5 µL of DNA template. A *M. tuberculosis* strain, H37Rv, and *M. africanum* strain ATCC 25420 were included in PCR runs as reference strains.

A 548-bp region of the oxyR locus was amplified by PCR using the primer sequences described by Sreevatsen et al. (11). After an initial denaturation step of 4 min at 95°C, amplification was performed for 30 cycles with the following parameters: 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s. A final elongation step was performed for 5 min at 72°C. For katG, a primer set, 5′-TGCTGGCGCTTGGCAATACA and 5′-GCCGCGCTTGTCGCTACC, was designed to amplify a 429-bp region encompassing codon 463. With the exception of an anneal-

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ing temperature of 60°C, the amplification parameters for katG were identical to those described for oxyR. The amplified products were purified by using QIAquick spin columns (Qiagen Inc., Valencia, CA) and subjected to dRhodamine Terminator Cycle Sequencing (Applied Biosystems, Foster City, CA) as recommended by the manufacturer. The cycle sequencing reactions were analyzed on an ABI 377 DNA sequencer, and the sequences aligned with those derived from *M. tuberculosis* strain H37Rv and *M. africanum* strain ATCC 25420.

For case histories, see Table 1. The five isolates were identified as *M. tuberculosis* complex by a positive result with the Gen-Probe AccuProbe system and by the presence of IS*6110* insertion sequences. When spoligotyping was performed on isolates from cases A through E, all were found to have the *M. africanum* signature spoligotype, as described by Viana-Niero et al. (4). The results of spoligotyping are shown in the Figure.

The five isolates were *M. africanum*, based on several criteria shown in Table 2. Susceptibility of the strains to PZA, nitrate reduction, production of niacin, and the presence of guanosine at *oxy*R285 were incompatible with identification of the isolates as *M. bovis*. The RD9 deletion was shown for all five isolates and the *M. africanum* reference strain, ATCC 25420, by 1) the presence of a ≈480-bp band on the agarose gel when the PCR was performed with the flanking primers and 2) the absence of a ≈375-bp band when the PCR was performed with the internal primers. The RD9 deletion was incompatible with identification of the isolates as *M. tuberculosis*, and CTG at katG463 suggested that the isolates were not *M. tuberculosis*, since most isolates of this species have CGG at this site (7).

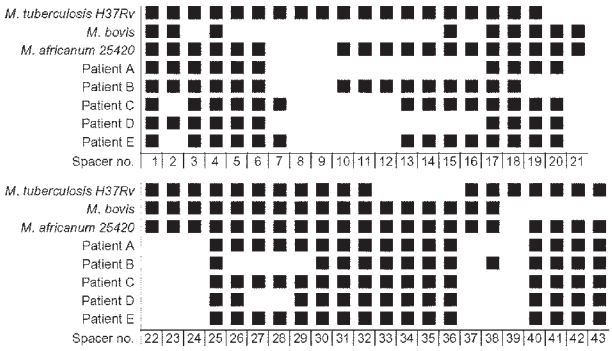

Figure. Spacer oligonucleotide typing (spoligotyping) results for cultures from patients A through E, together with representative patterns from stock cultures of *Mycobacterium tuberculosis* complex species. Spoligotypes for patients C and E are identical.

Conclusions

David et al. (12) and Frothingham et al. (13) reported that some isolates of *M. africanum* are resistant to PZA and fail to accumulate niacin, distinguishing them from most strains of *M. tuberculosis*, while other isolates are susceptible to PZA and produce niacin. These five isolates of *M. africanum* were susceptible to PZA, and three out of the five tested positive for niacin production and nitrate reduction (the remaining two cultures had lost viability and were

Table 1. Clinical characteristics of patients with *Mycobacterium africanum* disease

Patient	Demographic characteristics	Signs and symptoms	Clinical characteristics	Treatment[a]	Outcome
A	25-y-old black South African man with recent travel to West Africa	3 months' productive cough, weight loss, and low-grade fever	Large left upper lobe infiltrate on chest radiograph	INH, rifampin, PZA, and ethambutol by directly observed therapy for 6 months	Resolution of symptoms and improvement of chest radiograph
B	34-y-old black West African woman recently arrived in U.S. with metastatic carcinoma, treated with chemotherapy 1 year before presentation.	2 months' cough, fever, night sweats, fatigue	Right lower lobe cavitation, streaky infiltrates bilaterally on chest radiograph, normocytic anemia	INH, rifampin, PZA, and ethambutol by DOT	Sputum culture negative after 2 weeks of therapy. Only modest improvement in chest radiograph. Returned to West Africa after 4 months DOT
C	26-y-old black South African, immigrated to U.S. 2 y before diagnosis	Unknown	Cavitary lesion on chest radiograph	INH, rifampin, PZA, and ethambutol by DOT for 6 months	Sputum culture and smear negative, chest radiograph improved
D	26-y-old Vietnamese man, immigrated to U.S. 6 y before diagnosis. No history of travel to Africa or African contacts with tuberculosis	2 weeks' productive cough, positive PPD	Unknown	Capreomycin, ofloxacin, PAS, clofazamine, and cycloserine for 2 y by DOT	Sputum smear and culture converted to negative, symptoms resolved. Free of disease 2 y after therapy completed
E	27-y-old U.S.-born black man with no known risk factors for tuberculosis infection. No history of travel to Africa or African contacts with tuberculosis	2 months' anorexia and weight loss; 1 month of cough and night sweats; 1 week of fever and abdominal pain	Normal chest radiograph results. Pancreatic cyst and fullness of left psoas muscle on CT scan	INH, rifampin, PZA, and ethambutol for 10 months by DOT	Symptoms resolved. Free of disease 1 y after therapy completed

[a]INH, izoniazid; PZA, pyrazinamide; DOT, directly observed therapy; CT, computerized tomography, PPD, purified protein derivative; PAS, para-aminosalicylic acid.

Table 2. Phenotypic and molecular characteristics of *Mycobacterium africanum* isolates and country of birth for five patients

Patient	No. of copies of IS*6110* (bands)	Spoligo-typing pattern missing spacers 8,9,39?	*Oxy*R 285[a]	Codon *kat*G463 sequence[b]	Presence of RD9 deletion?	PZA susceptibility[c]	Niacin production	Nitrate reduction[d]	Major site of disease	Birth country
A	10	Yes	G	CTG	Yes	S	+	3+	Lung	South Africa
B	3	Yes	G	CTG	Yes	S	ND[e]	ND	Lung	Ivory Coast
C	9[f]	Yes	G	CTG	Yes	S	ND	ND	Lung	South Africa
D	11	Yes	G	CTG	Yes	S	+	4+	Lung	Vietnam
E	9	Yes	G	CTG	Yes	S	+	3+	Pancreas	USA

[a]Isolates of *M. bovis* do not have G at this site.
[b]CTG is found in *M. bovis* and *M. africanum*, but not in most strains of *M. tuberculosis.*
[c]S, susceptible; PZA, pyrazinamide.
[d]3+ and 4+ reactions are considered positive.
[e]ND, not done (because the culture lost viability).
[f]Restriction fragment length polymorphism patterns for isolates from patients C and E were identical.

not available for this testing). This rendered the cultures phenotypically indistinguishable from *M. tuberculosis* (12–14). For each of the reported cases, the signature *M. africanum* spoligotype pattern described by Viana-Niero et al. (4) was supported by the single nucleotide polymorphisms characteristic of *M. africanum* and by the RD9 mutation that characterizes members of the tuberculosis complex other than *M. tuberculosis*.

At present, the Microbial Diseases Laboratory plans to spoligotype all strains of *M. tuberculosis* complex. Clinical features and drug susceptibility were not distinctive for *M. africanum* isolates, nor were all associated with patients who came from Africa. No connection with a source case from Africa was found for patients D or E. However, since spoligotyping will be routine, noting the signature *africanum* spoligotype may be worthwhile. This spoligotype may indicate an increased chance that the patient may have acquired the *M. tuberculosis* complex infection in Africa or possibly from an African source case.

Dr. Desmond has worked at the Microbial Diseases Laboratory, California Department of Health Services, since 1990. His research interests include laboratory methods for detecting drug resistance in *Mycobacterium tuberculosis* and applications for molecular strain typing in the mycobacteriology laboratory.

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Rapidly Progressive Dementia Due to *Mycobacterium neoaurum* Meningoencephalitis

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Dementia developed in a patient with widespread neurologic manifestations; she died within 5 months. Pathologic findings showed granulomatous inflammation with caseation necrosis, foreign body–type giant cells, and proliferative endarteritis with vascular occlusions. Broad-range polymerase chain reaction identified *Mycobacterium neoaurum* as the possible pathogen. Central nervous system infection by *M. neoaurum* may result in rapidly progressive dementia.

A few dementing illnesses are characterized by rapid cognitive decline and early emergence of neurologic signs. Causes include malignancy, vascular disorders, autoimmune disorders, and infections. We describe a patient in whom dementia associated with cerebellar, pyramidal, extrapyramidal, and bulbar manifestations developed; the patient died within 5 months. Postmortem examination showed chronic granulomatous meningitis and arteritis. Broadband polymerase chain reaction (PCR) identified the presence of DNA from *Mycobacterium neoaurum*.

The Case

A 63-year-old woman was assessed for rapid functional decline over 2 months, with cognitive impairment, multiple falls, incontinence, and dependence for most basic daily activities. She could ambulate no further than a few meters despite assistance. Medical history included stroke 10 months previously with mild residual left-sided weakness, depression, rheumatoid arthritis, and hypertension. She smoked heavily, did not abuse alcohol or injection drugs, and had never received blood products. Medications included prednisone 5 mg daily, paroxetine, amlodipine, clopidogrel, estrogen, calcium, vitamin D, bromazepam, and acetaminophen. She was white and had spent all her life within the Great Lakes area of southern Ontario. She had once worked in a plant manufacturing leather components for automobiles, and her husband worked briefly as a meat packer. She had no family history of neurologic illness. She did not consume raw meat and had no contact with livestock but used sheep manure in her garden.

Cognitive testing showed impaired abstract thinking, memory, and attention but no affective or psychotic disturbance. She was afebrile with no nuchal rigidity. Speech production was reduced, aprosodic, and dysarthric. Cranial nerves were otherwise unremarkable. She exhibited hypomimia, limb rigidity with intermittent cogwheeling, and left arm dysmetria. No tremor or startle myoclonus was noted. Power was moderately reduced in all limbs. Reflexes were brisk with bilateral spontaneous ankle clonus of both ankles. Bilateral plantar responses were extensor. She could not walk unaided.

The patient was referred to a consultant and hospitalized. Complete blood count, blood urea nitrogen, creatinine, electrolytes, calcium, alkaline phosphatase, bilirubin, thyroid-stimulating hormone, and serum B12 were normal. Serum albumin was 28 g/L (normal 33–48 g/L), serum glutamic oxaloacetic transaminase 54 U/L (normal 5–40 U/L), serum glutamic pyruvate transaminase 58 U/L (normal 5–40 U/L), and erythrocyte sedimentation rate 74 mm/h. Serum antinuclear antibodies, extractible nuclear antibodies, antineutrophil cytoplasmic antibodies, Venereal Disease Research Laboratory test, and complement levels were unremarkable. Serologic tests for hepatitis B and C and enzyme-linked immunosorbent assay for HIV were negative. Electroencephalograph (EEG) demonstrated intermittent irregular slow delta waves in the right frontal and left temporal regions but no biphasic or triphasic waves. Magnetic resonance scan of the brain showed multiple areas of remote and recent infarction involving right frontal cortical and subcortical regions, pons and cerebellum, and right parasagittal frontal cortex. A diagnosis of recurrent strokes was made, but she continued to decline after discharge to a rehabilitation hospital. She became mute, immobile, and in need of complete assistance. She freely aspirated and was hypoxic. A lumbar puncture obtained shortly before her death showed cerebrospinal fluid (CSF) glucose of 4.8 mmol/L (normal 2.5–4.4 mmol/L), total protein of 0.33 g/L (0.15–0.60 g/L), and 8 x 10^6 lymphocytes/L with no malignant cells. Bacterial, mycobacterial, and fungal stains and cultures and viral cultures were negative. Protein 14-3-3 (Centre for Research in Neurodegenerative Disease at the University of Toronto) was present in the CSF.

The brain weighed 1,530 g. The circle of Willis was normal with no atheroma or occlusions. External examina-

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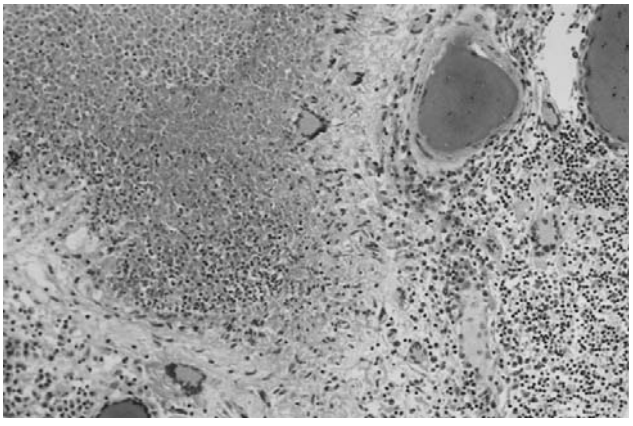

Figure 1. Meningeal infiltrate with caseation necrosis surrounded by giant cells. A nearby vessel is surrounded by a mononuclear cell infiltrate (occipital lobe, x250, stained with hematoxylin and eosin–Luxol-fast blue).

tion showed focal areas of yellow exudate on the convexities and multiple bilateral infarcts affecting the cortices, pons, thalamus, middle temporal gyrus, and putamen. Histologically, some infarcts were bland while others were associated with a thick exudate characterized by granulomatous inflammation with caseation necrosis and foreign body–type giant cells (Figure 1). Numerous vascular occlusions with no atheroma were noted (Figure 2). A proliferative endarteritis was observed. In some areas, the process appeared resolved with extensive leptomeningeal fibrosis. Ziehl-Nielsen and auramine rhodamine stains failed to demonstrate mycobacteria. Gram stains, fungus stains, and prion protein immunostains (3H4, Prionics, Zurich, Switzerland) were negative. A moderate degree of diffuse arteriolosclerosis was observed. An occluded Charcot-Bouchard aneurysm was identified in the right temporal cortex.

Broad-range PCR identification was performed on frozen brain tissue by using the modified method of Heritz et al. (1). PCR (PerkinElmer, Foster City, CA) and lysis buffers were pretreated with 8-methoxypsoralen at a final concentration of 25 µg/mL and exposed to long-wave (360 nm) UV light for 15 min to destroy preexisting DNA contaminants. Tissue samples (approximately 2 mm^3) were lysed in 50 µL 1x PCR buffer containing 1% polyoxyethylene 10 lauryl ether and 200 µg/mL proteinase K at 56°C overnight. Lysate, in 1- and 5-µL aliquots, was used as template for PCR amplification with universal primers for bacterial 16S rDNA (515FPL and 13B [2]). PCR mixture without lysate was used as a negative control, and a cloned 16S rDNA amplification product was used as a positive control (20 ng DNA). The PCR protocol involved a 3-min hot start at 95°C, followed by addition of primers at 50 pmol per 50-µL mixture. Thirty cycles of PCR were performed (94°C, 45s; 55°C, 45s; 72°C, 1 min) followed by a final extension step at 72°C for 10 min, and the amplification products were stored at 4°C. The PCR products were separated on a 1.5% agarose gel and visualized on a UV transilluminator after being stained with ethidium bromide (0.5 g/mL) for 15 min at room temperature. Amplification products were cloned into the plasmid vector pCR2.1TOPO (Invitrogen, Burlington, Ontario). Cloned products were fingerprinted by endonuclease digestion with *Taq*I or *Rsa*I, and unique clones were sequenced by using dye terminator methods (ABI PRISM BigDye Terminator Cycle Sequencing Kit, Applied Biosystems, Foster City, CA). The resulting DNA sequences were identified by comparison with the nonredundant database at the National Center for Biotechnology Information (3) and showed a match of 577 of 581 nt (99%) to *M. neoaurum* (GenBank accession no. AF268445).

Conclusions

M. neoaurum, first described in 1972, belongs to the *M. parafortuitum* complex of the genus *Mycobacterium* (4). *M. neoaurum* is a rapidly growing scotochromogen that is found in soil. Eight cases of human infection from *M. neoaurum* have been described in the English-language literature (5–11). Six of these cases were associated with central venous catheters, one was associated with intravenous drug use, and another involved urinary isolation of *M. neoaurum* during an investigation of a catheter-associated urinary tract infection. None of these cases was fatal. CNS infection has not been previously described.

Several features of this case suggest that infection caused by *M. neoaurum* was responsible. First, the patient declined rapidly, which is unusual in common dementias. Second, a mild CSF lymphocytic pleocytosis is consistent with an infectious or inflammatory CNS disorder, though rare occurrences have been reported in pathologically confirmed Creutzfeldt-Jakob Disease (CJD) (12). Third, histologic examination demonstrated a caseating granulomatous

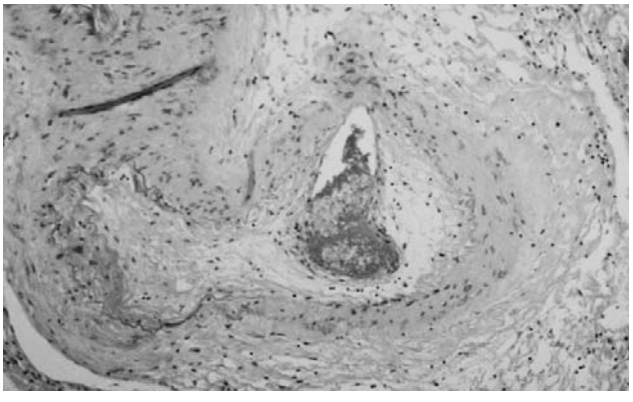

Figure 2. Proliferative endarteritis. The artery is stenotic and partially occluded by fibrous tissue. The residual lumen is almost completely occupied by recent thrombus (frontal lobe, x160, stained with hematoxylin and eosin–Luxol-fast blue).

process with accompanying endarteritis, consistent with mycobacterial infection. *M. neoaurum* is relatively difficult to culture; therefore, inability to cultivate the organism or visualize it on Ziehl-Nielsen and auramine rhodamine stains is not unexpected.

The patient had no known risk for parenteral exposure to bloodborne pathogens and was not apparently immunocompromised. Although the epidemiology of her case suggests mycobacterial infection through direct exposure, the mode of acquisition is speculative.

The patient was diagnosed with CJD. In patients with rapidly progressive dementia, probable CJD may be diagnosed if myoclonus or typical EEG tracings are present, according to Brown et al. (13). Possible CJD is diagnosed when rapidly progressive dementia is associated with a movement disorder or periodic EEG activity. Masters proposed that probable CJD may be diagnosed in patients with rapidly progressive dementia, biphasic or triphasic waves on EEG, and at least two of myoclonus, visual or cerebellar symptoms, pyramidal or extrapyramidal signs, and akinetic mutism (14). Possible CJD is diagnosed when typical EEG findings are absent. Recent efforts to improve diagnostic accuracy for CJD have focused on measuring CSF protein 14-3-3. Thought to reflect neuronal injury, reported sensitivities and specificities of CSF protein 14-3-3 for CJD in patients with rapidly progressive dementia are 84%–96% and 87%–100%, respectively (15–21). The Masters' criteria have been revised to reclassify possible CJD as probable if CSF protein 14-3-3 is present (18). In the case described, revised Masters' criteria for probable CJD and Brown criteria for possible CJD were met. The false-positive rate of CSF protein 14-3-3 for CJD can be as high as 12%, however, with other causes including vascular disorders, infectious encephalitis, anoxia, malignancy, and even Alzheimer's, Lewy body, and frontotemporal dementias (15,17–21). In this patient, the presence of CSF protein 14-3-3 likely reflected ischemic injury mediated by the proliferative arteritis.

In summary, we have described a case of rapidly progressive dementia with prominent neurologic features attributable to chronic granulomatous meningitis and arteritis. Despite negative stains and cultures, the identification of DNA from *M. neoaurum* suggests that this case may represent the first reported CNS infection from this organism, as well as the first documented fatality. The absence of any clear mode of infection or predisposing risk factors for developing such a devastating infection is unusual. This case highlights the difficulties in achieving a causative diagnosis in patients with rapidly progressive dementia. CSF protein 14-3-3 does not entirely rule out potentially treatable causes, and in this case an angiogram would likely not have been able to differentiate inflammatory from infectious vasculitis. More definitive diagnostic methods are required, including a possible role for PCR analysis of CSF samples, as well as earlier consideration of biopsy.

Acknowledgements

We thank the Canadian CJD Surveillance System, Division of Health Care Acquired Infections, Health Canada, for its support; R. Tersigni for her administrative assistance; I. Frohn and Bryan Korithoski for their technical support; and the family of Isabel Loth for their contribution towards the completion of this report.

Dr. Heckman, an internist and geriatrician, is currently a clinical scholar at McMaster University. His primary research interests are in cognitive and functional outcomes of cardiovascular disease in the frail elderly.

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Hemolytic Uremic Syndrome Incidence in New York[1]

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A comparison of New York's traditional communicable disease surveillance system for diarrhea-associated hemolytic uremic syndrome with hospital discharge data showed a sensitivity of 65%. *Escherichia coli* O157:H7 was found in 63% of samples cultured from hemolytic uremic syndrome patients, and samples were more likely to be positive when collected early in illness.

Diarrhea-associated hemolytic uremic syndrome (HUS) is a major cause of acute renal failure in children (1). Various studies have demonstrated that Shiga toxin–producing *Escherichia coli* are the etiologic agents in most cases of diarrhea-associated HUS (2,3). In the United States, *E. coli* O157:H7 is the most common Shiga toxin–producing *E. coli*, causing an estimated 73,480 infections (4); HUS develops in 3%–15% of patients soon after the onset of diarrhea (5,6).

The risk factors associated with the progression of *E. coli* O157 to HUS include very young or old age, elevated leukocyte count, and use of antimicrobial treatment or antimotility agents (7–11). Little is known about the clinical features that could identify patients at high risk for HUS. In New York, both *E. coli* O157 infection and diarrhea-associated HUS became reportable in July 1994. Since then, the annual incidence rate for *E. coli* O157 per 100,000 population has ranged from 1.6 in 1995 to 8.5 in 1999; the annual incidence rate for diarrhea-associated HUS per 100,000 population has ranged from 0.1 in 1995 to 0.2 in 1999.

HUS is a severe disease that results in hospitalization. Our surveillance and hospital discharge data were used to evaluate the HUS surveillance system, estimate the number of diarrhea-associated HUS cases, and study the epidemiologic and clinical features of HUS in New York.

The Study

A confirmed case of HUS was defined as the acute onset of anemia with microangiopathic changes (i.e., schistocytes, burr cells, or helmet cells) on peripheral blood smear, acute renal injury (i.e., hematuria, proteinuria, or elevated creatinine level), and a low platelet count (platelets <150,000/mL) within 3 weeks of an acute diarrheal illness. A probable case of HUS was defined as the acute onset of anemia with microangiopathic changes, acute renal injury, and a low platelet count without a history of diarrhea in the preceding 3 weeks; or hemolytic anemia without confirmed microangiopathic changes, acute renal injury, and a low platelet count within 3 weeks of an acute diarrheal illness (12).

Hospital admission notes, laboratory reports, and discharge summaries were requested for patients who had HUS (International Classification of Diseases, 9th Revision, Clinical Modification [ICD-9-CM] code 283.11) listed as their primary or any secondary diagnosis for 1998 and 1999. All medical charts were reviewed, and patients were classified as having a confirmed, probable, or undetermined HUS case. Persons with multiple hospitalizations were counted only once. The records from case reports and medical charts were matched by last name, first name, and date of birth. The capture-recapture method was used to evaluate the completeness of HUS reporting and to estimate the "true" number of HUS cases and the 95% confidence interval (13).

Data from medical charts regarding demographic characteristics (age, sex, race, month of admission, hospital length of stay), clinical features at admission (vomiting, fever, bloody stool), and antimicrobial therapy were extracted for all confirmed or probable case-patients. Laboratory variables were obtained for patients within 7 days before and 3 days after the HUS diagnosis and included the lowest hematocrit, lowest platelet count, highest blood urea nitrogen (BUN), and highest creatinine concentrations. The Fisher exact test was used to determine the proportion of demographic and clinical characteristics among HUS patients with or without stool isolates for *E. coli* O157:H7, and the two-tailed Student *t* test was used to determine the significance of differences between their mean laboratory variables.

Forty-five HUS case-patients reported to the New York communicable disease surveillance system for 1998 through 1999 were listed as being hospitalized; the medical charts of 44 of these patients were available for review. We requested 542 medical records that had a primary or secondary discharge diagnosis listed as HUS during the same period; 421 (78%) charts were received. After chart review, 234 records were from New York State residents, excluding New York City, and 201 patients remained for

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[1]This paper was presented at the International Conference on Emerging Infectious Diseases, held March 24–27, 2002, in Atlanta, Georgia, USA.

analysis after excluding duplicate records. Forty-nine patients had confirmed HUS, 10 patients had probable HUS, and 142 patients either had incomplete information to determine HUS status or did not have HUS. Among these 142 patients, 9 patients had no laboratory reports, 23 patients had renal failure and anemia with microangiopathic changes but no diarrhea; and 110 patients had no renal failure or no microangiopathic changes. Stool samples from 12 of these 142 patients were cultured for *E. coli*; none was positive.

HUS surveillance data were matched against hospital discharge data by last name, first name, and date of birth. Thirty-three confirmed and 5 probable cases were in both systems, 4 confirmed and 3 probable cases were only in the surveillance system, and 16 confirmed and 5 probable cases were only in the hospital system. The "true" total number of confirmed or probable HUS cases in New York was estimated to be 70 (95% confidence interval [CI] = 63 to 75), and the sensitivity of our surveillance system for reporting HUS was 65%.

The demographic, clinical features, and laboratory results of 53 confirmed and 12 probable diarrhea-associated HUS cases from either data system were studied. The average annual incidence was significantly greater among children <5 years of age (relative risk [RR] 17, 95% confidence interval [CI] 8.9 to 32) than among patients 15–64 years of age and was greater among female than male patients (RR 2.1, 95% CI 1.2 to 3.8) (Table 1). The median length of hospital stay was 11 days. Review of clinical features indicated that 62 (95%) had diarrhea; 46 (71%) had bloody stools. While hospitalized, 20 (31%) patients received antimicrobial agents and 19 were treated before HUS developed. Thrombocytopenia (platelets <150,000/mL) was present in 90% of the patients, and 85% had hemolytic anemia with microangiopathic changes on peripheral blood smear. HUS patients ≤15 years of age were more likely to have fever (64%) and bloody stools (82%) than those >15 years of age (19% and 54%, respectively).

Six patients (9%), ages 3 to 89 years, died while hospitalized; two of these deaths were outbreak related. All six patients had bloody diarrhea and blood transfusions, leukocyte counts >18,000, BUN levels >63, and serum creatinine levels >2.3. Samples from five of six were culture positive for *E. coli* O157:H7.

Of the 65 confirmed or probable HUS patients, 54 (83%) had their stool or urine tested for *E. coli* O157:H7; 34 (63%) of these patients had positive results (33 stools and 1 urine). Of the 34 culture-confirmed patients, 30 were reported to the surveillance system, and 4 patients received a diagnosis only in the hospital system. A significantly higher proportion of patients with samples culture-positive for *E. coli* O157 patients had shorter mean durations from diarrhea onset to specimen collection, and lower BUN values within 7 days of admission than did culture-negative *E. coli* O157 patients (Table 2). A higher proportion of patients with outbreak-related HUS cases had positive *E. coli* O157 cultures (13/15, 87%) than those with nonoutbreak-related cases (21/50, 42%).

Table 1. Demographic and clinical characteristics of 65 patients with confirmed or probable hemolytic uremic syndrome (HUS), New York, 1998–1999

Characteristic	No. of patients (N = 65)		
Demographic features	No. (incidence/100,000)		
Age (in years)			
<5	28 (2.0)		
5–14	11 (0.4)		
15–64	17 (0.1)		
>65	9 (0.3)		
Sex			
Male	20 (0.2)		
Female	45 (0.4)		
Race			
White	57 (0.3)		
Black	3 (0.2)		
Asian	1 (0.2)		
Outcome	No.(%)		
Alive	59 (91)		
Dead	6 (9)		
Outbreak-associated	No. (%)		
Yes	15 (23)		
No	50 (77)		
Clinical features	No.(%)		
Diarrhea	62 (95)		
Protein in urine	52 (80)		
Blood in urine	52 (80)		
Any transfusion	50 (77)		
Blood in stool	46 (71)		
E. coli isolated	34 (52)		
Fever	30 (46)		
Vomiting	28 (43)		
Hemodialysis	19 (29)		
Treated with antimicrobial agents	20 (31)		
Laboratory test results	Mean	Median	Range
Creatinine (mg/dL)	4.1	3.3	0.3 – 13.2
Blood urea nitrogen (mg/dL)	72.0	69.0	8.0 – 146.0
Platelet/1,000	55.0	38.0	5.0 – 298.0
Leukocytes/1,000	20.1	19.6	7.0 – 47.0
Hematocrit (%)	21.9	21.3	9.4 – 35.5

Conclusions

Most surveillance systems for communicable disease reporting are passive. The use of hospital discharge records is a conventional method to verify the completeness of reporting. A population-based study that used hospital data without reviewing medical charts estimated that 47% of hospital discharge data were reported to public health sur-

Table 2. Characteristics of HUS cases by *Escherichia coli* O157:H7 culture isolation status, New York, 1998–1999

Characteristics	*E. coli* O157:H7 culture		
	Positive (n = 34)	Negative (n = 20)	p value
Mean age at admission (y)	23.6	23.7	0.99
Mean length of hospital stay (days)	13.4	12.6	0.71
Mean duration from diarrhea onset to specimen collection date (days)	4.1	6.0	0.06
Median duration from admission date to specimen collection date (days)	0	1.0	0.02
Mean BUN (mg/dL)	62.1	82.6	0.05
Mean creatinine (mg/dL)	3.6	4.8	0.24
Mean platelet count ($/10^3$)	50.9	53.8	0.81
Mean leukocyte count ($/10^3$)	18.5	21.4	0.23
% outbreak related	38%	5%	0.01
% blood in stool	82%	65%	0.15
% reported to surveillance	88%	55%	0.01
% microangiopathic change	79%	100%	0.03
% urinary tract infection	3%	20%	0.04

[a]HUS, hemolytic uremic syndrome; BUN, blood urea nitrogen.

veillance (14). Our disease surveillance system's sensitivity of 65% in identifying diarrhea-associated HUS was higher than that figure but also reinforces the importance of medical chart audit. A possible contributing reason for the low sensitivity may be the difficulty in identifying confirmed HUS cases. To enhance surveillance, New York, as a FoodNet site, extended the chart review for hospitalized patients <18 years of age statewide in whom HUS was diagnosed.

Seven of the 12 probable HUS case-patients had *E. coli* O157:H7 isolated without evidence of erythrocyte fragmentation, suggesting that hospitals may not report probable cases. A study to match *E. coli* O157:H7 cases from our surveillance system against HUS diagnosed cases from a hospital discharge data system and to review all case-patients hospitalized with *E. coli* O157:H7 is under way. Results should identify the possible diarrhea-associated HUS cases missed by our surveillance system.

This study showed that female patients had higher incidence rates for HUS; the incidence rates of *E. coli* O157:H7 showed no differences by gender. *E. coli* O157:H7 was isolated from stool or urine samples from 34 (63%) of 54 confirmed and probable HUS patients who were cultured, a finding that demonstrates that *E. coli* O157:H7 is the predominant organism related to diarrhea-associated HUS in New York. Other studies have had similar findings (15,16). Samples from HUS patients cultured on or before the hospital admission date had a higher recovery rate of *E. coli* O157 from stool cultures (78%) than those cultured after hospital admission (41%). Patients who did not have *E. coli* O157:H7 isolated were cultured later in illness (6 days) than those from whom this pathogen was recovered (4 days), a finding that reemphasizes the importance of obtaining early cultures for microbiologic diagnosis (17).

HUS associated with *E. coli* O157:H7 infection is a serious illness with a 5%–10% death rate (5). Prompt followup of cases using pulsed-field gel electrophoresis molecular methods has increasingly led to identifying food sources and removing contaminated products from the food distribution system. Complete and rapid reporting of cases is a crucial component of public health prevention activities.

Acknowledgments

We thank Nancy Spina for reviewing hospital medical charts.

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Multidrug-resistant *Salmonella* Typhimurium Infection from Milk Contaminated after Pasteurization

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An outbreak of multidrug-resistant *Salmonella enterica* serotype Typhimurium infections occurred in Pennsylvania and New Jersey. A case-control study implicated pasteurized milk from a dairy, and an inspection indicated the potential for contamination after pasteurization. Dairy cattle are the likely reservoir, and milk may be an important vehicle of *Salmonella* transmission to humans.

Pasteurization, or heat treatment, of milk is an important milestone in public health that contributed to dramatic declines in many infectious diseases. Despite the important public health gains achieved, outbreaks associated with pasteurized milk continue to occur (1–3). We describe a recent outbreak associated with pasteurized milk.

The Study

On April 13, 2000, the Pennsylvania State Department of Health notified the Centers for Disease Control and Prevention (CDC) of an increase in *Salmonella enterica* subspecies *enterica* serotype Typhimurium. Active surveillance for *Salmonella* group B and serotype Typhimurium was initiated, and health officials in Maryland, Delaware, New Jersey, and New York were notified. Isolates were sent to public health laboratories for confirmation and serotyping and to New Jersey and CDC for antimicrobial susceptibility testing by MIC with broth microdilution and molecular subtyping by pulsed-field gel electrophoresis (PFGE) and phage typing (4,5). To identify and compare isolates from cows during the time of the outbreak, we contacted personnel at the *Salmonella* Reference Center at the University of Pennsylvania.

Stool samples from 93 persons yielded *S.* Typhimurium (76 in Pennsylvania and 17 in New Jersey). Dates of illness onset ranged from March 6 to April 19 (Figure). The median age of patients was 9 years (range 3 months–88 years), and 51 (55%) were male. PFGE was performed on 44 of 93 isolates from patients. Of the 44 isolates, 26 were pattern A, 1 pattern B, 5 pattern C, 7 pattern D, and 1 each for pattern E, F, G, H, and I. The three dominant patterns (A, C, and D) formed a complex of highly related strains (1 band difference, 1 band shift, or both). We defined the 38 isolates with patterns A, C, and D as outbreak-related strains. The outbreak-related strains were unique when compared to the other 3,469 *S.* Typhimurium PFGE patterns in the PulseNet database (CDC, Atlanta, GA). Isolates identified as outbreak-related strains were all phage type 21. Of the 16 isolates tested for antimicrobial resistance, 12 (7A, 1B, 1C, 3D) were resistant to ampicillin, kanamycin, streptomycin, sulfamethoxazole, and tetracycline (AKSSuT), 3 (1A, 2C) were AKSSu, and 1 (A) was ASSu resistant. Two of the three *S.* Typhimurium isolates obtained from dairy cows during the same time period were also outbreak-related strains.

We conducted 11 interviews with patients who had recent onset of disease; during these interviews, infection through milk consumption became a leading hypothesis. In the case-control study, a case was defined as an outbreak-related isolate of *S.* Typhimurium in a resident of Pennsylvania or New Jersey with illness onset between

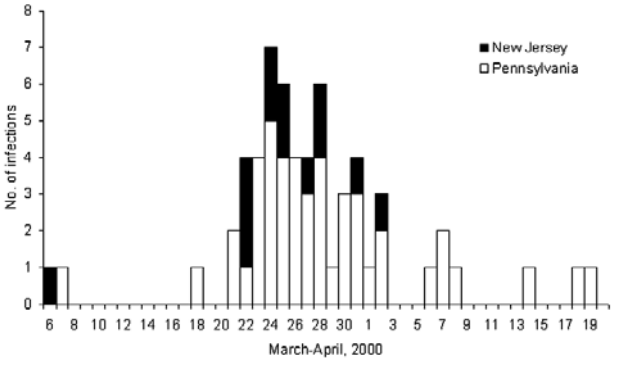

Figure. Dates of illness onset among persons with *Salmonella enterica* subspecies enterica serotype Typhimurium infection, Pennsylvania and New Jersey, March–April, 2000

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March 1 and April 21. If more than one person in a household met the case definition, we interviewed the person with earliest onset. Two controls per case were selected by random digit dialing and matched to patients by area code and three-digit telephone prefix and age group. Using a questionnaire administered over the telephone, we asked about exposures in the 5 days before patient's illness onset. Parents responded for children.

We interviewed 23 (61%) of the 38 eligible case-patients and 37 controls; they did not differ with respect to age or sex (median age 5 years vs. 5 years, p = 0.8; percent male: 70% vs. 54%, p = 0.2). Dates of diarrhea onset were from March 20 to April 19. Twenty-two (96%) patients reported diarrhea, 17 (74%) bloody diarrhea, 20 (87%) abdominal cramping, 16 (70%) fever, and 8 (35%) vomiting. In addition, 6 patients (26%) were hospitalized, and 9 (39%) were treated with antimicrobial drugs (amoxicillin [2 patients], ciprofloxacin [2 patients], sulfamethoxazole [1 patient], unknown agent [4 patients]).

Ill persons were 22 times more likely to drink milk from dairy plant X than controls (20 of 23 patients vs. 13 of 37 controls, matched odds ratio = 23, 95% confidence interval = 2.7 to 184.5). Odds of infection increased with drinking larger amounts of milk from dairy plant X (p = 0.0008). Other risk factors, including handing reptiles or consuming chicken, undercooked eggs, sprouts, or unpasteurized milk or juice were not associated with illness. Although Philadelphia patients were excluded from the case-control study for logistic reasons, seven were residents or employees of an independent living facility where more than 80% of the milk received was from dairy plant X.

During the last 2 weeks of April, state and federal agencies visited dairy plant X. The plant purchased raw milk from over 59 different farms. Finished product was distributed in seven counties in eastern Pennsylvania as well as to Delaware and New Jersey. Dairy plant X was regularly inspected every 3 months by the Pennsylvania Department of Agriculture. The most recent inspection before the outbreak was in January 2000; no problems were reported.

According to our review of time and temperature pasteurization charts, pasteurization was adequate during the time of the outbreak. Our review of in-house microbial testing results from January 3 and April 17 identified 13 instances where the standard plate count was elevated and 9 instances where coliforms were elevated. The highest standard plate count was 120,000/mL on April 4, and the highest total coliform count was reported as >100/mL on April 14 and 17; both occurred in skim milk. According to the Pasteurized Milk Ordinance, the standard plate count should not exceed 20,000/mL, and the coliform count should not exceed 10/mL.

Inspectors from the Food and Drug Administration (FDA) found violations of sanitary standards that could have resulted in contamination of products after pasteurization. These violations included evidence of excessive condensation throughout the processing and packaging area. High humidity and excessive condensation could have produced droplets that fell into open containers. In addition, several machines leaked raw milk onto the floor, and raw skim milk was held in a silo at >10°C (standard: 7.2°C).

Sixty-six milk samples with production dates from April 3 to 20 were collected and tested by the Pennsylvania Department of Agriculture. None grew *Salmonella*; two grew *Escherichia coli*. None of 26 environmental samples collected April 28 grew *Salmonella*. All 172 finished milk samples collected in May and July were negative for *Salmonella* and had <1 μg/mL phosphatase, indicating all were adequately pasteurized.

A review of records at dairy plant X identified 14 employees who were absent between March 20 and April 20. Three (21%) had a gastrointestinal illness with onset March 20, March 26, and one unknown; a stool sample from one yielded the outbreak-related strain of *S.* Typhimurium. All reported drinking finished products produced at the plant in the 5 days before their illness onset.

Conclusions

We describe a large, multistate outbreak of multidrug resistant *S.* Typhimurium infections linked to pasteurized milk. *Salmonella* likely contaminated the containers or milk contact surfaces after pasteurization because of environmental conditions in the plant, likely originating in *Salmonella*-contaminated raw milk. Two dairy cow isolates of *S.* Typhimurium obtained during the outbreak period were outbreak-related strains, which suggests that these strains were circulating in Pennsylvania dairy herds. Although federal agencies asked for access to the farms that provided the milk to the plant, these farms were not identified, which prevented further preplant investigation.

S. Typhimurium resistance type AKSSuT that caused this outbreak appears to be emerging and raises similar concerns to those that surround *S.* Typhimurium definitive type 104 (DT104) (6). Antimicrobial drugs are commonly used to treat persons with salmonellosis and can be life saving in severe infections. Antimicrobial resistance can limit treatment options, can contribute to treatment failure, and is associated with increased deaths (7).

The importance of pasteurized milk as a source of salmonellosis is largely unknown. We reviewed the published literature and identified 12 outbreaks in the United States between 1960 and 2000 that were associated with pasteurized milk (Table). Of the 12 outbreaks, seven were caused by contamination after pasteurization, and five were caused by *Salmonella*. Although published reports are relatively rare, outbreaks may not be recognized for several

Table. Review of pasteurized milk outbreaks in the United States, 1960–2000

Date	Location (ref)	Pathogen	Setting	Total no. ill (confirmed)	Mechanism of contamination
Nov 1966	Florida (8)	*Shigella flexneri* type 2	Community	97 (97)	After pasteurization
Jul–Aug 1975	Louisiana (9)	*Salmonella* Newport	Military base/community	49 (49)	Unknown
Sep–Oct 1976	New York (10)	*Yersinia enterocolitica* O:8	School	38 (38)	After pasteurization
Oct 1978	Arizona (11)	*S.* Typhimurium	Community	23 (23)	After pasteurization
Jun–Jul 1982	Tennessee, Arkansas, Mississippi (12)	*Y. enterocolitica* O:13, 18	Community	172 (172)	Unknown
Jun–Aug 1983	Massachusetts (13)	*Listeria monocytogenes* 4b; phage type 2425A	Community	49 (40)	Unknown
Apr 1984	Kentucky (14)	*S.* Typhimurium	Convent	16 (16)	Inadequate pasteurization
Mar–Apr 1985	Illinois (1)	*S.* Typhimurium	Community	>150,000 (>16,000)	After pasteurization
Mar–Apr 1986	Vermont (15)	*Campylobacter jejuni* O:2, 36	School	33 (8)	Inadequate pasteurization
Jul 1994	Illinois (2)	*L. monocytogenes* 1/2b	Picnic	45 (11)	After pasteurization
Oct 1995	Vermont, New Hampshire (3)	*Y. enterocolitica* O:8	Community	10 (10)	After pasteurization
Mar–Apr 2000	Pennsylvania, New Jersey	*S.* Typhimurium, phage type 21, R-type AKSSuT	Community	93 (38)	After pasteurization

reasons. Milk is an extremely common exposure, which makes reporting exposure to milk likely, obscuring an association. Typhimurium is one of the most common serotypes of *Salmonella*, which make detecting outbreaks more difficult in the absence of subtyping. Several factors may enhance detection of pasteurized milk-associated outbreaks: a very focal illness event, illness caused by an unusual strain, a method for subtyping surveillance strains, heavy contamination of the product, and a local brand of milk.

The outbreak we report led to immediate changes in dairy plant X. The plant hired an outside consultant and addressed FDA's immediate concerns. In addition, the Pennsylvania Department of Agriculture began to integrate plant employee training with routine inspections. And finally, as routine inspection regimens did not prevent the outbreak, the findings from this investigation prompted FDA to move up its scheduled review of the state milk regulatory program. Although the results of this review are not available to federal authorities or the public, Pennsylvania's milk control program now satisfies all of the FDA criteria for certification.

Current milk standards are designed largely to safeguard against a failure or breakdown in the process of pasteurization. Our review of milk-borne outbreaks suggests that inadequate pasteurization is a relatively uncommon event compared to contamination after pasteurization. Additional regulatory emphasis on post pasteurization monitoring, such as coliform and standard plate count, may be needed for adequate protection.

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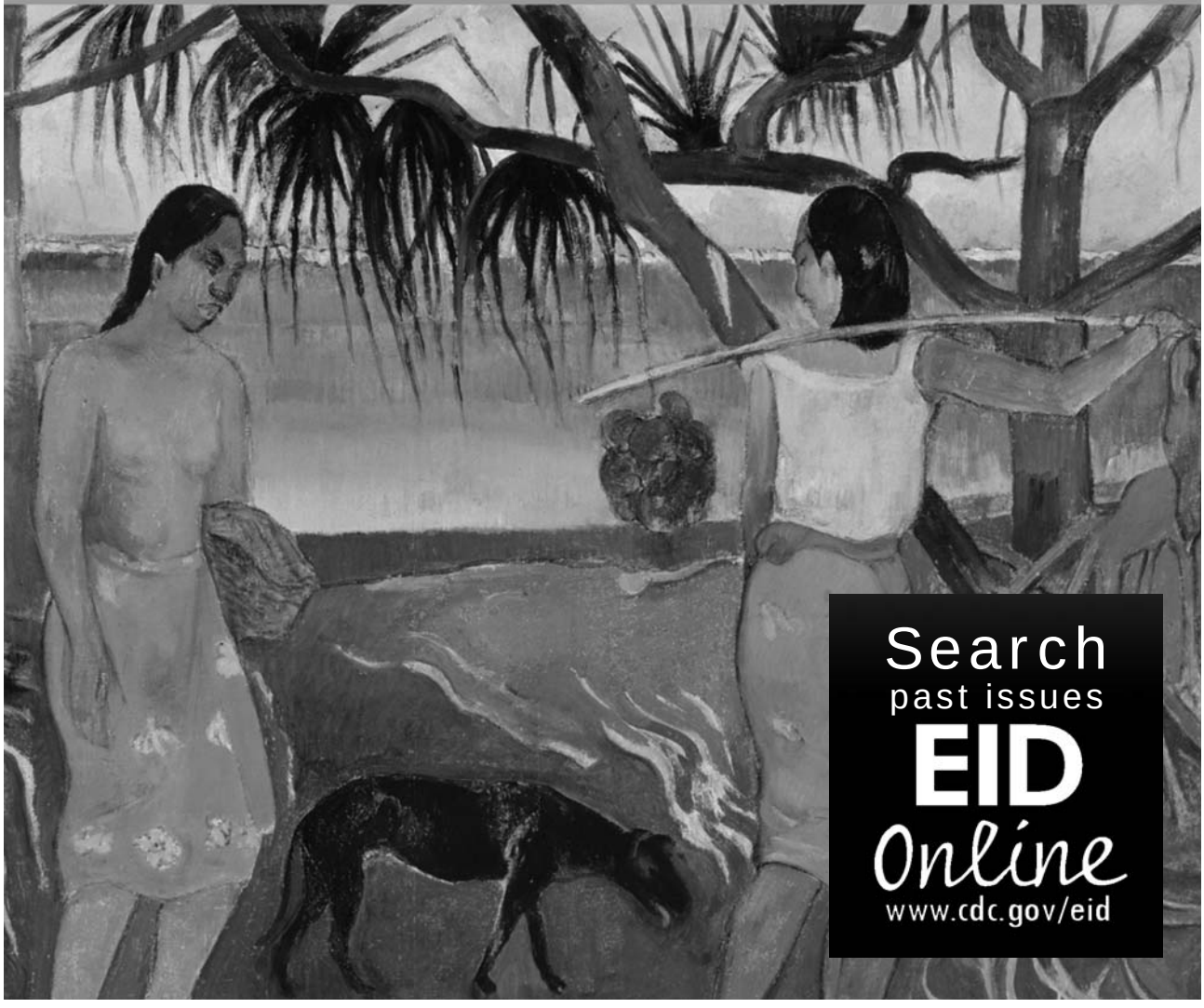

Neisseria meningitidis W135, Turkey

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We describe the first case of *Neisseria meningitidis* W135 meningitis in Turkey. The strain was genotypically unrelated to the clone (W)ET-37, isolated from Hajj pilgrims in 2000.

The Case

A previously healthy 20-year-old serviceman experienced chills, headache, and vomiting 2 days before being admitted to the hospital in March 2003. On physical examination, neck stiffness, Kernig sign, Brudzinski sign, and temperature of 40°C were noted. The patient's cerebrospinal fluid (CSF) was turbid with increased protein and pressure; leukocyte count was 4,500/μL. CSF culture grew *Neisseria meningitidis* in 24 hours. The strain was serogrouped as W135 by specific antiserum (Difco, Sparks, MD) in Hacettepe Medical School, Turkey, and confirmed by the Centers for Disease Control and Prevention (CDC, Atlanta, GA). Blood culture results were negative, and the patient had no petechial rash. He was treated with high-dose cefotaxime (3 g every 6 hours for 14 days) and made a full recovery.

For this isolate, both disk-diffusion and E-test methods using cefotaxime, penicillin, tetracycline, and ciprofloxacin were performed according to the criteria defined by the British Society for Antimicrobial Chemotherapy (1,2). Both methods were performed on Iso-Sensitest agar (Oxoid, Basingstoke, UK), supplemented with 5% defibrinated horse blood and nicotine adenine dinucleotide (Sigma, Taufkrichen, Germany). The isolate was susceptible to all of the antimicrobial agents (Table). The serviceman did not attend the Hajj and had no history of travel or contact with returning pilgrims.

Table. Susceptibility testing results of the isolate by E-test and disk-diffusion method

Antimicrobial agent tested	MIC (μg/mL)	Zone diameter (mm)
Penicillin	0.050	30
Cefotaxime	0.016	30
Tetracycline	1.000	23
Ciprofloxacin	0.002	32

Conclusions

To the best of our knowledge, *N. meningitidis* W135 meningococcal disease has never been reported in Turkey. One W135 isolate from an asymptomatic carrier was reported in a child in 2001 (3). Globally, W135 strains are often isolated after intensive vaccination campaigns against serogroup A and C meningococci have been implemented (4). This patient's vaccination certificate confirmed that he had received a bivalent (A+C) meningococcal vaccine 2 months earlier, at the beginning of his military training period. Turkish military vaccination campaigns have used the A+C polysaccharide vaccine successfully for a decade.

Multilocus enzyme electrophoresis, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing, multilocus DNA fragment typing, and sequencing the 16S rRNA gene are new genotypic approaches to characterize *N. meningitidis* strains (5). This isolate was genotyped by using PFGE and 16S sequencing at CDC; both methods showed that it was a different subtype than the one associated with the Hajj pilgrimage in 2000 and 2001 (Figure).

In Turkey, most of the population is Muslim, and approximately 150,000 pilgrims travel annually to Saudi Arabia for the Hajj. During the pilgrimage in 2000 and 2001, an international outbreak was caused by a previously rare meningococcal serogroup W135 clone, (W)ET-37, possibly because conditions during the pilgrimage facilitate person-to-person transmission of meningococci (6,7). For the Hajj season of 2002 and 2003, all Turkish pilgrims received a quadrivalent meningococcal polysaccharide vaccine (Mencewax ACWY, SmithKline Beecham, Genval, Belgium). Although the quadrivalent meningococcal vaccine can protect persons against disease attributable to W135, it does not prevent them from becoming asymptomatic carriers, and therefore the vaccine may not prevent transmission to unvaccinated household contacts (7,8).

A recent study in the United States (9) showed that 0.8% of 727 returning pilgrims in 2001 were W135 carriers, although none had been on departure. To our knowledge, the rate of pilgrims returning to Turkey as W135 carriers has not been studied. On the basis of W135 transmission rates and epidemiologic data, we estimated the risk of an unvaccinated contact who had acquired W135 developing invasive meningococcal disease to be 1 case per 70 infections (7). In Singapore, disease usually developed within 14 days of a person's contact with Hajj pilgrims, and no cases occurred 2 months after the end of Hajj pilgrimages (7). In Mauritius, a small tropical island in the Indian Ocean, one case of meningococcal disease caused by W135 occurred in a girl 3 months after her father returned from the Hajj pilgrimage; however, the virus could not be cultured, and it was not shown to be related to

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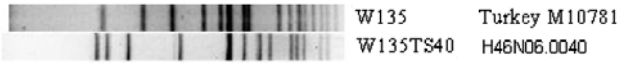

Figure. PFGE images of the case isolate (top) and the clone ET-37 (bottom).

the Hajj strains (10). The case we report here occurred approximately 50 days after most Turkish pilgrims returned, which suggests that it was unrelated to the Hajj.

Although our patient had no history of travel or contact with a returning pilgrim, we investigated possible associations with the Hajj. However, PFGE results indicated that our patient's strain was not closely related to the (W)ET-37 clone associated with the Hajj and may be unique to Turkey. Similarly, Jolley et al. from the Czech Republic have also reported sequence types of W135 other than (W)ET-37 (11). Additional investigation will be required to produce a database of well-documented Turkish cases. After the outbreaks in 2000 and 2001, many European countries reported additional cases of W135 meningitis in persons with no history of pilgrimage or contact with a returning pilgrim.

Since quadrivalent meningococcal vaccine does not prevent asymptomatic infection and therefore may not prevent returning pilgrims from transmitting W135 to unvaccinated household contacts, prophylactically administering antimicrobial agents should be considered to reduce the risk for transmission. Any decision to administer chemoprophylaxis to all returning pilgrims should depend on the rate of transmission of W135 infection from asymptomatic carriers to contacts after future pilgrimages. This case also showed the continuing need for administering quadrivalent meningococcal vaccine in the community. W135 meningococcal disease appears to be an emerging problem that should be investigated epidemiologically. This case confirmed the need to further study meningococcal carriers in order to build a national database and help make decisions on prophylaxis.

Acknowledgments

We thank Leonard W. Mayer and Tanja Popovic for confirming the serogroup and PFGE genotype of the *Neisseria meningitidis* W135 isolate.

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Dead Crow Reports and Location of Human West Nile Virus Cases, Chicago, 2002

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During the summer and fall of 2002, an epidemic (223 cases) and epizootic of West Nile virus infections occurred in Chicago. Retrospective spatial analysis demonstrated that age-adjusted human case rates were three times higher inside geographic areas with high early-season crow deaths than outside these areas.

West Nile virus (WNV) activity was first identified in North America in New York City in 1999 (1). In each subsequent year, the WNV epidemic and epizootic have spread across the continent (2), as new susceptible human, avian, and equine populations are exposed to the virus.

During previous seasons of WNV transmission in most affected areas of the United States, the infection and deaths of crows (*Corvus brachyrynchos*) have preceded the detection of human infections. Tracking sightings of dead crows is advocated as an integral aspect of WNV activity surveillance systems (3,4). The utility of dead crow surveillance in predicting the intensity or geographic extent of subsequent human epidemics, however, is uncertain (5).

During the summer of 2002, an epizootic and epidemic of WNV infections occurred in Chicago, a city of 2.9 million residents and an area of 231 square miles. We conducted a retrospective study to assess the spatial relationship between the locations of dead crow sightings reported early in the transmission season and the residences of persons subsequently reported to have cases of WNV infection.

The Study

Human cases of aseptic meningitis and encephalitis are reportable conditions in Illinois. During 2002, the Illinois Department of Public Health determined WNV-positive cases by using its own laboratory as well as outside laboratory services. Upon notification by the state health department of a WNV-positive result in a Chicago resident, the Chicago Department of Public Health conducted an epidemiologic investigation of the case.

Human cases of WNV infection were classified as WNV meningoencephalitis or WNV fever. Cases in patients with acute onset of signs and symptoms consistent with meningitis or encephalitis were defined as WNV meningoencephalitis. Cases without acute onset of signs and symptoms consistent with meningitis or encephalitis were classified as WNV fever.

In Chicago, citizens may call the city's nonemergency hotline to report dead bird sightings or to request pickup and disposal of a dead bird. For each such call, operators record the date of the call, the type of bird reported, and the dead bird's street location.

The first serologic confirmation of human WNV infection in a Chicago resident was reported on August 12, 2002. By the end of 2002, a total of 223 cases of WNV infection were reported in Chicago residents, with onsets of illness occurring from July 18 to October 7. The human epidemic peaked during the week ending August 31 (week 35). The first dead crow sighting recorded by the nonemergency hotline occurred on July 1; by October 19, a total of 3,837 dead crow sightings at distinct addresses had been reported through the hotline. These reports peaked during the week ending August 17 (week 33) (Figure 1).

Spatial analysis was used to assess the relationship between the locations of dead crow sightings reported before the first human case of WNV infection was detected and the reported residence of human WNV case-patients. Spatial analysis was conducted by using ArcView Spatial Analyst version 1.1 in ArcView version 3.2, and maps were generated with ArcView version 8.2 (all software: Environmental Systems Research Institute, Inc.,

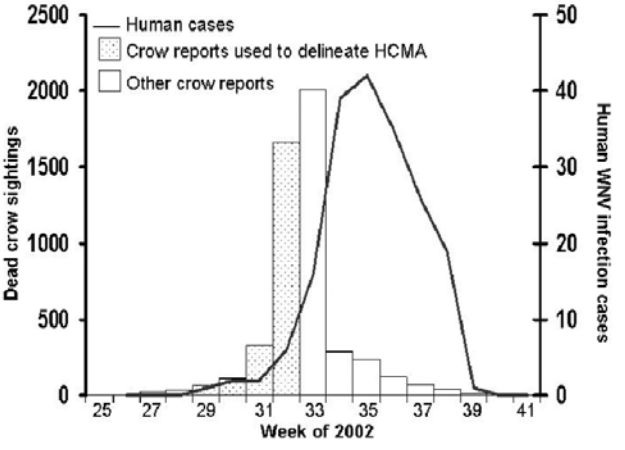

Figure 1. Dead crow sightings reported to the Chicago nonemergency hotline, by week of report, and human cases of West Nile virus infection, by week of illness onset, Chicago, June 16 (beginning of week 25) through October 12 (end of week 41), 2002. HCMA, high crow-mortality area.

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Redlands, CA). Dead crow sightings reported through the hotline from July 1 through August 11 were geocoded and mapped. Smoothed dead crow densities were generated by using kernel estimation to calculate density and a bandwidth radius of 1 mile. The kernel function was used to describe mathematically the geographic density of crow deaths by assigning a value to a point on the map; the value is a function of that point's proximity to surrounding crow deaths (6). The dead crow density values were reclassified into deciles. All map areas with a density value classified in the top nine deciles were delineated and designated as high crow-mortality areas. Such areas were then expanded to conform to census tract boundaries.

Human cases of WNV infection were geocoded and mapped by using the case-patient's reported street address of residence. Human case rates were calculated on the basis of the 2000 census population within and outside the high crow-mortality areas. To control for age, the data were stratified by age group (0–24 years, 25–49 years, 50–64 years, ≥65 years); effect modification and confounding were assessed; and the rates within and outside the high crow-mortality areas were compared by calculating a Mantel-Haenszel weighted incidence ratio with Greenland-Robins 95% confidence intervals (CI) in EpiInfo version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA). Because WNV fever case-patients were often seen as outpatients and their inclusion could have introduced diagnostic access bias into the findings, the analysis was conducted a second time, with patients limited to those meeting the WNV meningoencephalitis case classification.

Of 3,837 dead crow sightings reported through week 32, a total of 3,833 (>99%) had address information sufficient for geocoding. Home address data were obtained for 219 (98%) of the 223 Chicago case-patients. The high crow-mortality area covered 124 square miles (54% of Chicago's surface area), and 179 (82%) of the geocoded case-patients resided within it's the city's boundaries (Figure 2A). The crude case rate for WNV infection was 10.8/100,000 inside the high crow-mortality areas compared with 3.2/100,000 outside (Mantel-Haenszel weighted incidence ratio 3.0, 95% CI 2.1 to 4.2). For WNV meningoencephalitis only (n = 166), the crude case rate was 7.8/100,000 inside the high crow-mortality areas compared to 2.9/100,000 outside (Figure 2B) (Mantel-Haenszel weighted incidence ratio 2.3, 95% CI 1.6 to 3.4). The spatial association was not confounded by age group, nor did the strength of association differ significantly by age group.

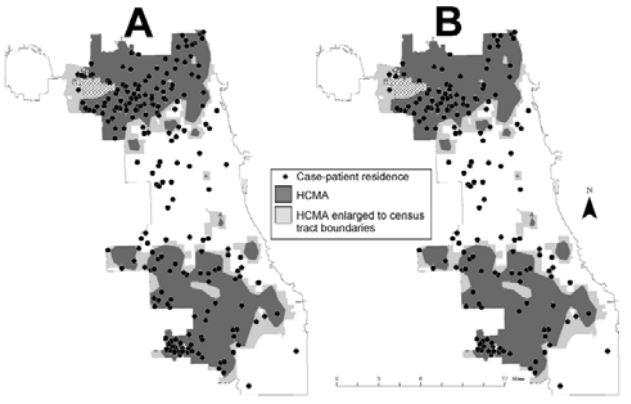

Figure 2. Chicago map with high crow-mortality areas (HCMAs) and reported residences of A) West Nile virus (WNV)-infected case-patients, or B) WNV meningoencephalitis case-patients (WNV fever cases excluded), 2002.

Conclusions

We identified a spatial association between early-season crow deaths and residences of WNV-infected case-patients in Chicago. The findings indicate that a functioning dead-crow surveillance system could assist in prioritizing geographic areas for mosquito-control interventions, such as risk communication and larval and adult mosquito control. These interventions could occur before, or concurrently with, the identification of WNV-infected case patients in a given transmission season.

The success of dead crow surveillance systems depends in part upon public participation, and the Chicago non-emergency hotline—a simple, well-recognized, and well-publicized means of collecting dead crow data—was an essential prerequisite to this analysis. One potential source of bias that accompanies these data, nonetheless, is the self-selection of persons who report crow deaths. Residents of certain neighborhoods might be more inclined to notify their local government upon seeing a dead bird than those in other neighborhoods.

WNV-associated avian deaths have historically preceded human illness, and in Chicago, the epizootic peaked approximately 2 weeks before the human epidemic. Real-time analysis of incoming dead crow reports might be especially important because of the time lag between illness onset, confirmatory testing for WNV infection, report to the local health authority, and initiation of an epidemiologic case investigation to identify and adequately describe WNV-related human illness. In Chicago in 2002, the first case of WNV infection in a resident was reported on August 12, 13 days after the patient's onset of illness.

Whether a similar spatial association between early-season crow deaths and residences of WNV-infected case-patients will be evident in future seasons is unknown, as an estimated 81% of the Chicago-area crow population is thought to have died in 2002 (7). Nonetheless, our experience highlights the potential utility of spatial analysis of dead crow reports in prioritizing geographic areas for mosquito-control interventions.

Acknowledgments

We thank S.I. Gerber and J.R. Fernandez.

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Community-acquired Methicillin-resistant *Staphylococcus aureus* among Military Recruits

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We report an outbreak of 235 community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) infections among military recruits. In this unique environment, the close contact between recruits and the physical demands of training may have contributed to the spread of MRSA. Control measures included improved hygiene and aggressive clinical treatment.

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first recognized in the 1960s and has since become a well-known cause of nosocomial infections (1). Recently, MRSA has been reported with increasing frequency outside healthcare settings (2–5). Community-acquired outbreaks have been reported in a variety of populations, including prison inmates (3,4), players of contact sports (6,7), children in daycare (8), and crewmembers of a naval ship (9). These groups do not possess the risk factors traditionally associated with MRSA infection, namely, recent hospitalization, dialysis, residence in a long-term care facility, or intravenous drug use (1,2). We report an outbreak of community-acquired MRSA infections among recruits at a large military training facility in the southeastern United States.

The Study

The training facility where the outbreak occurred had a recruit population that fluctuated from 3,500 to 7,000. A case-patient was defined as a recruit with a clinically recognized skin or soft-tissue infection and a positive MRSA culture from the site of infection. Laboratory records showed that from October 2000 to July 2002, 47 culture-confirmed MRSA infections occurred among recruits (Figure 1). During this period, the monthly incidence of MRSA did not exceed two cases per 1,000 recruits (Figure 2). However, from August to December 2002, 235 MRSA cases occurred. During the outbreak period, the monthly incidence rates ranged from 4.9 to 11 cases per 1,000 recruits.

Of the case-patients, 209 (89%) were men. This percentage paralleled the overall male recruit population in 2002 (88% male). Although information on the specific age of infected recruits was not available, all recruits at this facility were 17–25 years of age. Most infections occurred on an extremity (73.7%), most commonly the lower leg (16.0%) and the knee (13.9%) (Table).

To investigate what aspects of training might be associated with transmission, cases were sorted by week of training when illness was diagnosed (Figure 3). Data on training week was available for 143 (61%) of the outbreak patients. The rise in cases during weeks 1–5 suggests that transmission increased with time in training. Of the cases, 86% occurred during weeks 6 to 12 but did not seem to be associated with any single event. Moderate increases occurred during weeks 6 and 7 (rifle range training) and week 11, which included the "crucible," a 54-hour strenuous field exercise and final test before graduation. These weeks include important milestones for recruits, and some may have delayed seeking medical care until after completing these steps.

Medical records from 20 patients were randomly selected and reviewed during the investigation. These patients included 18 men and 2 women, 17–24 years of age. The diagnoses included abscesses (15 patients), cellulitis (2 patients), and folliculitis (3 patients). The antimicrobial agents most commonly prescribed for initial treatment were dicloxacillin (6), levofloxacin (5), and

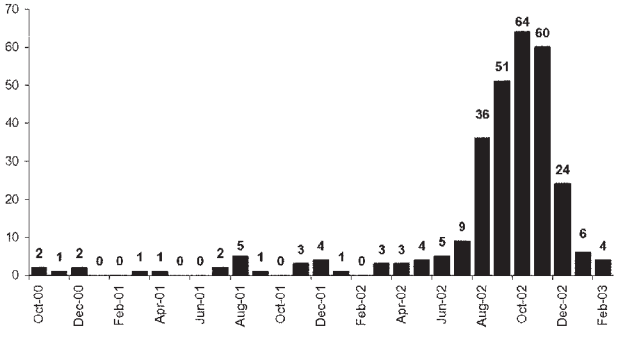

Figure 1. Methicilin-resistant *Staphylococcus aureus* cases in recruits.

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[1]Views expressed are those of the authors and are not approved by or representative of the Bureau of Medicine and Surgery, the Department of the Navy, or the Department of Defense.

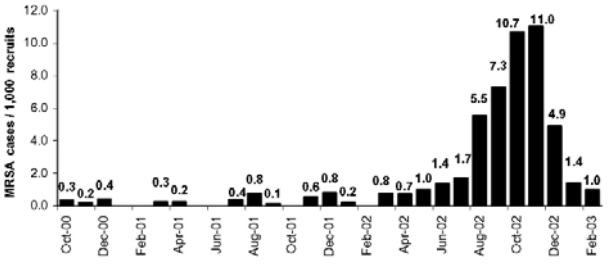

Figure 2. Methicilin-resistant *Staphylococcus aureus* (MRSA) incidence.

ciprofloxacin (4). No patients had a history of hospitalization within the previous year, although one patient had been treated with levofloxacin for pneumonia 2 weeks before.

Nasal screening was conducted to identify carriers and determine the colonization rate among staff members permanently assigned to the training facility. Anterior nasal swabs were obtained from 874 workers who had direct contact with recruits, including medical, dental, and laboratory personnel, drill instructors, barbers, and other ancillary staff. Of these, 24 (2.7%) were colonized with MRSA.

Through interviews with healthcare providers, laboratory personnel, and recruits, investigators found that most patients did not display established risk factors for MRSA (history of chronic medical conditions, hospitalization or surgery within the previous year, history of drug use, or recent use of an antimicrobial agent). Also, the MRSA isolates were sensitive to many commonly used outpatient antimicrobial agents, including trimethoprim/sulfamethoxazole and clindamycin.

No recent lapses in recruit hygiene training or practices had occurred. Recruits were afforded daily time for showering, cleaning, and personal hygiene. However, this time was limited, perhaps leading to deficient hygiene practices among some recruits (i.e., inadequate showering, infrequent handwashing, sharing towels and other personal items).

In November 2002, facility personnel implemented an array of control measures with an emphasis on improving hygiene and treatment regimens. Based on existing recommendations for preventing MRSA transmission in healthcare settings (10), antibacterial soaps and hand sanitizers were placed at all recruit sinks, and investigators recommended that hand washing be conducted as frequently as possible. All recruits were issued personal bottles of antibacterial hand sanitizer for use when soap and water were not readily available. Daily showers of adequate duration were enforced, and sharing personal items such as towels and razors was prohibited.

In addition, local healthcare providers were alerted to the presence of MRSA among recruits. Culturing of lesions was encouraged. Patients were treated with the following regimen aimed at eliminating both MRSA infection and nasal carriage: oral rifampin and minocycline for 10 to 14 days, nasal mupirocin twice daily for 10 days, and Hibiclens washes. (Trimethoprim/sulfamethoxazole could be substituted for minocycline.) Finally, preventive medicine staff conducted biweekly surveillance for MRSA cases by using laboratory records.

Table. Anatomic site of MRSA infection

Site[b]	No. patients	(%)
Lower extremity	86	(44.3)
Thigh/hip	15	(7.7)
Knee	27	(13.9)
Leg	31	(16.0)
Ankle	2	(1.0)
Foot	11	(5.7)
Upper extremity	57	(29.4)
Axilla	8	(4.1)
Arm	20	(10.3)
Elbow	13	(6.7)
Forearm	7	(3.6)
Hand	9	4.6
Head	4	(2.1)
Face	4	(2.1)
Neck	3	(1.5)
Torso	3	(1.5)
Back	9	(4.6)
Buttocks	12	(6.2)
Inguinal	1	(0.5)
Genital	4	(2.1)
Urine	1	(0.5)
Sputum	1	(0.5)
Tissue, unspecified	9	(4.6)

[a]MRSA, methicillin-resistant *Staphylococcus aureus*.
[b]Site unknown for 41 patients.

The outbreak ended in December 2002, shortly after interventions were implemented. The actual number of cases as well as the incidence (cases per 1,000 recruits) declined by more than half in December 2002 and decreased further in January and February 2003 (Figures 1 and 2).

Conclusions

This large outbreak demonstrates the threat of MRSA in a close-contact environment such as recruit training. Our findings are consistent with community-acquired, rather than nosocomial, MRSA infection (1–3,6). MRSA is spread by direct contact, most often through the hands of an infected or nasally colonized person (3). Several recent community-acquired MRSA outbreaks have involved comparable close-contact environments (3,8,9). Spread of community-acquired MRSA has also been associated with prolonged physical contact between sports participants (6,7). Activities such as hand-to-hand combat training, life-saving, and team skill-building exercises involve similar physical contact between recruits.

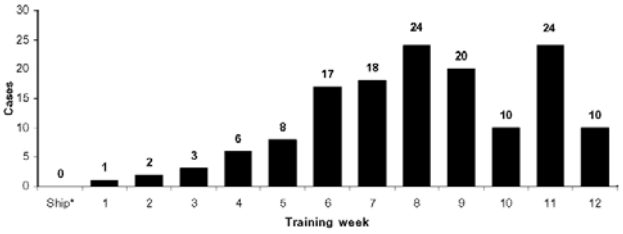

Figure 3. Methicilin-resistant *Staphylococcus aureus* cases by week of training. *Recruits arrive at the facility during ship week and undergo medical and administrative in-processing.

The physical nature of recruit training is another factor that may have contributed to this outbreak. Recruits often have minor cuts or abrasions that increase the risk of developing skin infections. Such injuries would be expected during physically demanding activities such as running, hiking, and negotiating obstacle courses. Indeed, most MRSA infections occurred on exposed surfaces such as arms, legs, and knees.

The growth and transmission of methicillin-sensitive *S. aureus* (MSSA), and accordingly MRSA, are increased in humid environments (11). The number of cases increased during warmer months, a time when recruits have more exposed skin surfaces. This may increase their risk for superficial wounds as well as contact with other recruit's skin surfaces. Furthermore, some recruits reported that their infections started as insect bites, also a seasonal problem.

The outbreak was unlikely to have originated from a single source. Cases occurred throughout the facility and were not localized to recruits who had contact with a particular instructor or other staff member. Further, the percentage of staff members at the facility who were found to be carriers was small and consistent with the 2%–3% MRSA carriage rates found in recent studies (9,12,13). Although how much contact these carriers routinely had with recruits is unclear, this small number of patients was not likely a major factor in spreading MRSA across so many different groups of recruits. In fact, the growing prevalence of MRSA in the general population (1,2,12,14) is an important concern because recruits may enter the military already colonized.

Maintaining good hygiene and avoiding contact with open skin lesions are the primary means to prevent the spread of MRSA infections (3,7). Although no recent changes in recruit hygiene had occurred that were directly responsible for the outbreak, the hygiene deficiencies noted in some recruits combined with the increased prevalence of MRSA were important contributing factors. Control measures were instituted to improve hygiene, including frequent hand washing and the use of antibacterial hand sanitizers. Similar measures have been implemented to control MRSA outbreaks in comparable settings (3,6,7,15).

Before August 2002, healthcare providers did not routinely obtain bacterial identification and sensitivities on skin infections, possibly delaying effective treatment in some cases. Once aware that MRSA was prevalent among recruits, healthcare providers improved treatment by culturing skin lesions whenever possible and prescribing appropriate antimicrobial agents for MRSA infections. Thus, the primary interventions used were recommending improved hygiene practices for recruits and implementing aggressive clinical protocols. These control measures, along with the onset of cooler weather, likely played important roles in ending the outbreak.

This outbreak occurred in a previously healthy military training population and was associated with close contact, limited opportunity for practicing good personal hygiene, warm weather, and physical stress. Reducing MRSA infections was related to implementing interventions to improve personal hygiene, aggressive evaluation and treatment of people with soft tissue injuries and infections, and cooler weather.

Acknowledgments

We thank John Gerhard for his support and assistance with the investigation, Joyce Lapa for editorial and writing assistance, Todd May for his support and assistance with data acquisition, and Joseph Cahill for his assistance with the investigation.

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Rift Valley Fever in Chad

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To evaluate the importance of human exposure to Rift Valley fever virus in Chad, investigations were carried out to determine specific antibody prevalence in domestic ruminants during the 2002 rainy season. Results highlighted recent, substantial, active transmission of this virus.

The virology laboratory of the unit of the Tropical Medicine Institute of the French Defense Medical Service (IMTSSA) investigated some cases of self-limiting nonmalarious febrile syndromes occurring among soldiers stationed in Chad. By using C6/36 and Vero cell lines, peripheral blood lymphocytes collected from two soldiers on duty in Chad during the 2001 rainy season were cocultured. Two strains of Rift Valley fever virus (RVFV) were isolated and identified by using indirect immunofluorescence, reverse transcriptase–polymerase chain reaction, and sequencing (1,2). To determine the potential for human exposure to RVFV, a seroprevalence investigation evaluated antibody prevalence in sheep, goats, and horned cattle among animals taken to slaughterhouses of N'Djamena and Abéché during the 2002 rainy season.

RVFV is a member of the genus *Phlebovirus*, family B*unyaviridae*, and was first isolated in Kenya in 1930. Transmitted by a wide variety of mosquitoes from several genera, the virus may cause abortion in pregnant livestock and high death rate in young animals. RVFV has caused influenzalike disease in humans, and it occasionally leads to more serious complications, such as retinitis, meningoencephalitis, or severe hemorrhagic fever with a high death rate.

In Chad, RVFV has never been officially recognized by either the World Health Organization or by the International Office of Epizooties. Nevertheless, three facts suggest that the virus is present in Chad's animal population. First, RVFV is generally thought to exist in the enzootic state in Central Africa in sheep and wild animals (3). Second, a study undertaken by the Pasteur Institute of Paris in collaboration with the EMVT (Département Elevage et Médecine Vétérinaire Tropicale du Centre de Coopération Internationale pour la Recherche en Agronomie pour le Développement) showed that 4% of sheep bred in Chad and Ethiopia had anti-RVFV neutralizing antibodies (4). Third, RVFV was identified in Sudan, Niger (5), and Nigeria, countries that border Chad.

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The Study

During the 2002 rainy season (August through October), within the slaughterhouses of the cities of N'Djamena (southwestern Chad between the 10th and 15th parallels, a few kilometers from Cameroon) and Abéché (220 km east of N'Djamena), blood samples were collected from randomly selected sheep, goats, and horned cattle (Table 1). According to the veterinary services of Chad's Ministry for Breeding, these animals were born and bred in Chad. Furthermore, they were gathered in parks a maximum of 3 days before slaughter. Sites were selected for their proximity to an area where French troops were deployed and also because the N'Djamena slaughterhouse, in particular, receives cattle from various parts of Chad.

Each sample was accompanied by information on the age of the animal (teeth examination), species, sex, and origin. Blood was centrifuged within 24 hours of collection. The serum was transferred into cryotubes and frozen at –80°C so that samples would arrive at IMTSSA for analysis still frozen (–20°C). Each sample was systematically tested for RVFV-specific immunoglobulin (Ig) G by using an enzyme-linked immunosorbent assay (ELISA). First, ELISA screening was done by using antigen capture (by mouse hyperimmune ascitic fluid) and detecting specific IgG in the diluted serum (1/500). The antigen used was a precipitate (polyethylene glycol 6000) of the supernatant of Vero cells infected with the RVFV clone 13. (This strain was isolated from a person in the Central African Republic [6] and is probably less dangerous than other strains for laboratory workers).

On the same ELISA plate as negative antigen, the serum was tested with Dugbe, a non-cross-reactive Nairovirus. All IgG-positive serum samples were retested for IgG (with a negative, noninfected Vero antigen) and IgM by using the M-antibody capture method. The most frequently used techniques for detecting anti-RVFV antibodies are immunofluorescence, plaque reduction neutralization assay, and immunoenzymatic assays (7). Because RVFV cross-reacts with many other phleboviruses (5), the choice of techniques used for this study was influenced by

Table 1. Number of blood samples per species and site

Animal	N'Djamena slaughterhouse	Abéché slaughterhouse	Total
Sheep	211	89	300
Goats	102	37	139
Horned cattle	99	15	114
Total	412	141	553

Table 2. Results of the cross-sectional investigation of Rift Valley fever antibody prevalence among sheep, goats, and horned cattle, Chad, 2002 rainy season[a]

Animal	Average age (y)	IgG+[b] animals (%)	Average age of IgG+ animals (y)	IgG+ animals age ≤1 y (% of those age ≤1 y)	% of animals killed in an area that were IgG+	Sex of IgG+ animals	IgG+ animals confirmed by WB	IgM+ animals (% IgG+)
Sheep	2.3	32/300 (10.7)	1.8	8 (12)	14.8% N, 1.1% A	7.5% of M, 12.8% of F	31	16 (50)
Goats	1.7	12/139 (8)	2.2	4 (6)	9% N, 5% A	6.7% of M, 10.8% of F	NA	4 (33.3)
Horned cattle	7.5	5/114 (4)	6.2	NA	5% N, 0% A	0% of M, 4.7% of F	NA	NA
Total	3.2	49/553 (8)	2.8	14 (10.5)	11% N, 2% A	4.4% of M, 11.4% of F	NA	20 (41)

[a]Ig, immunoglobulin; WB, Western blot; N, N'Djamena; A, Abéché; NA, not available.
[b]Serum positivity was established when the ratio between the optical density of the Rift Valley fever virus antigen and that of the Dugbe antigen was >3.5.

their sensitivity and specificity. Seroneutralization is described as the reference method for specificity (no cross-reaction with other phleboviruses) (7), but the need for cell culture makes it unsuitable for screening large numbers of serum samples (8). ELISA was preferred, since it is considered an efficient alternative in terms of sensitivity, specificity, and ease of use (7,8).

Serum samples were considered positive when the ratio between optical density associated with RVFV antigen and that associated with the Dugbe antigen was >3.5. Serum specimens demonstrating anti-RVFV IgG were validated in parallel by immunotransfer (Western blot) with a high threshold of positivity. Only serum samples containing both specific antibodies against the envelope glycoproteins (G1 and G2) and the nucleocapsid (NC) protein were considered positive. Comparative results between the two techniques confirm high specificity of ELISA (97% of the serum samples positive by ELISA were confirmed by Western blot). This high specificity has been described previously by crosschecking results with those of virus neutralization assay (8).

Conclusions

The relatively high prevalence of RVFV (Table 2) combined with the fact that 41% of IgG-positive animals are also IgM-positive (in cattle, these antibodies appear on the fourth day (9) after natural infection and persist for 2–6 months (10) underscore the seriousness of the situation in Chad. Indeed, many articles describe how domestic ruminants are an early and sensitive indicator of human epidemics (7) and how outbreaks of human infection are preceded by amplification cycles among animals (7).

The 1987 epizootic-epidemic in Mauritania was predicted by the Pasteur Institute of Dakar; by using a seroepidemiologic study among domestic animals, researchers showed that the virus had been circulating for at least 6 months in animal hosts and that an amplification cycle of the disease was in progress (11). We fear that, as occurred in Burkina Faso in 1987 (1), ecologic changes or climatic conditions favorable to vector proficiency (e.g., periods of intense rain associated with epizootic appearance in Kenya [12] and South Africa [13]) can increase, in areas where the virus circulates, antibody prevalence in animals and can lead to human cases (4). This risk appears even more important since human outbreaks are specifically preceded by an increase of antibody prevalence among animal populations. A study by the Pasteur institute of Paris showed that 4% of sheep bred in Chad and Ethiopia had anti RVFV antibodies (14), and these figures were repeatedly confirmed (4).

Data regarding origin (source and path) could not be collected for animals led to the N'Djamena slaughterhouse (all that was known was that they were born and bred in Chad) and are imprecise for those received at the Abéché slaughterhouse (local source not specified). Thus, charting the distribution of RVFV-positive animals and the geographic distribution of the virus is not possible. Nevertheless, the weak antibody prevalence in animals killed in the Abéché slaughterhouse should be noted, which allows us to conclude that this particular area is still isolated from RVFV.

Acknowledgments

We thank veterinary services of the Ministry for Breeding of Chad for their collaboration, the personnel of the medicosurgical center of the Kosseï base in N'Djamena who took part in this investigation, and J. Mosnier for his technical assistance.

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Bat Rabies, Texas, 1996–2000

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Bats submitted to the Texas Department of Health (1996–2000) were speciated and tested for rabies virus antigen by direct immunofluorescence microscopy. Antigenic analysis of rabies virus–positive specimens was performed with monoclonal antibodies against the nucleoprotein of the virus; atypical or unexpected results were confirmed by genetic analysis of nucleoprotein sequence.

Most information on bats as reservoirs for rabies virus (RABV) is obtained from animals submitted by the public to local health departments for rabies testing. These data are limited by the following factors: 1) most bat submissions are from a few species found around human dwellings and outbuildings; little is known about rabies in the >30 bat species whose habitats are restricted to forest, desert, and mountainous areas (1,2); and 2) few state laboratories identify their bat submissions to species, and fewer still have the resources to collect data on the incidence and prevalence of different antigenic and genetic variants of RABV (RABVV) (3).

The Texas Department of Health laboratory receives 600–1,300 bats each year for rabies testing. Approximately 11% of the bats submitted test positive for RABV. All are identified to species, and all RABV-positive specimens are typed with a panel of monoclonal antibodies (MAbs) to determine the antigenic variant of rabies. Samples from bat species uncommonly found rabid in Texas or from more common species infected with atypical virus variants are submitted to the Centers for Disease Control and Prevention (CDC) for nucleotide sequence analysis.

The objectives of this study were to determine the status of state surveillance for bat-associated rabies at the species level, assess the comparative characteristics of the antigenic and genetic variants of rabies in bats in Texas, and examine the need for bat speciation and genetic variant determination in assigning uniform variants of RABV.

The Study

All bats submitted to the Texas Department of Health Rabies Laboratory for RABV testing from 1996 to 2000 (n = 3,989) were used in this study. All bats were either identified upon receipt or frozen and saved for future speciation. A key based on external characteristics of adult bats from The Bats of Texas (4) was used to make initial determinations. Species identifications were confirmed by comparing specimen data with the more detailed descriptions in that book. All bats with uncertain identifications were taken to Bat Conservation International for clarification. Bats were shipped to Texas Tech University for species confirmation.

Brain tissues from RABV-positive bats were tested by direct immunofluorescence (Centocor, Malvern, PA; Chemicon, Temecula, CA) for their reaction with MAbs against the nucleoprotein of the RABV (5). MAbs were provided by CDC and have been used extensively to identify RABVV (1,6–10).

RNA in brain material was extracted with TRIzol, according to the manufacturer's instructions, then reverse transcribed and amplified by polymerase chain reaction using primers 10g and 304 (11). Amplicons purified by using the Wizard TM Minipreps DNA purification system (Promega, Madison, WI) were sequenced with the ABI PRISM DNA Sequencing Kit (PE Applied Biosystems, Foster City, CA), according to manufacturer's instructions. Automated fluorescence sequencing was performed on an Applied Biosystems 310 DNA sequencer (PE Applied Biosystem). Nucleotide sequence from a 302-bp region of the RABV nucleoprotein (bp 1175 to 1476) was aligned with Pasteur RABV, GenBank accession no. M13215 (12). Nucleotide sequence for Texas bat samples was compared to the 17 genetic lineages of RABV identified for bat samples in a CDC repository (GenBank accession nos. AF045166, AF394868-394888, and AY039224–39229) (13). A phylogenetic analysis of the sequence data was conducted by using the programs DNADIST, NEIGHBOR, SEQBOOT, and CONSENSE in the PHYLIP package, version 3.5 (14). Graphic representation of the phylogenetic analysis was obtained with the program TREEVIEW (15).

During the 5-year study period, 3,989 bats were submitted for RABV testing. More than 96% (n = 3,830) of all bats submitted from 1996 to 2000 were easily speciated; 159 (3.8%) were too decomposed, damaged, or immature for reliable identification to species or were inadvertently discarded before identification was complete. This dataset includes representatives from 19 of the 32 species found in Texas; also included are *Desmodus rotundus* from the Fort Worth Zoo and one or two species of fruit bats. *Tadarida brasiliensis* was the most common species submitted for testing, followed by *Lasiurus borealis*. Rare submissions include *Mormoops megalophylla*, *Myotis austroriparius*, *M. californicus*, *M. ciliolabrum*, *M. thysanodes*, *M. yumanensis*, *Antrozous pallidus*, and *Nyctinomops macrotis*. The

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prevalence of RABV in the submitted samples remained fairly constant; prevalence ranged from 8.9% in 1998 to 12.4% in 1997 with an average prevalence of 11%. Specimens from nine of the species tested positive for RABV. *L. cinereus* had the highest average positivity rate (26.3%) followed by *T. brasiliensis* (16.4%); this finding is in agreement with results of a recent study of the continental United States (16). *Nycticeius humeralis* had the lowest average positivity rate (0.7%) (Table 1).

MAb reaction patterns have been identified, and the complete N gene sequence is available from GenBank for the RABV associated with *T. brasiliensis*, *L. borealis*, *L. cinereus*, *L. intermedius*, and *Eptesicus fuscus* (Table 2 and Figure). Adequate material was available for examination of 407 of 416 rabies-positive samples (1996–2000) from these five bat species in Texas by antigenic analysis; 402 of 407 samples had reaction patterns that were expected for the species. Genetic analysis was used to confirm the antigenic typing result of 14 of the 407 samples typed by antigenic methods; other Texas samples included in the analysis are two samples not submitted to the Texas Department of Health, seven samples from 1986 to 1995, and 10 samples from bat species not commonly found rabid in Texas.

MAb reaction pattern 1 described the RABV in 331 of 332 samples from *T. brasiliensis*. MAb reaction pattern 2 was found in 1 of 332 samples, suggesting interspecific infection through contact with *L. borealis*; however, no RABV genetic material could be amplified from the *T. brasiliensis* sample displaying reaction pattern 2. Reference samples from *T. brasiliensis* collected in Texas in 1984, 1993, and 1998 clustered in lineage 31 with virus from *T. brasiliensis* collected across the range of these bats in the United States.

Reaction pattern 2 described the RABV in 45 of 46 samples from *L. borealis*. MAb reaction pattern 1 was found in 1 of 46 samples, suggesting interspecific infection through contact with *T. brasiliensis*; however, the finding was not confirmed by genetic analysis. This sample, txlb 5770, clustered in lineage 26 with reference samples of *L. borealis* collected in Texas in 1986 and virus from *L. borealis* collected elsewhere in the eastern United States.

Reaction pattern 3 described the RABV in 13 of 14 samples from *L. cinereus*. *L. cinereus* samples displaying reaction pattern 3 (n = 4) clustered in lineage 23 with *L. cinereus* samples collected across the range of these bats in the United States. One *L. cinereus* sample (txlc4259) differed in its reaction with the MAb panel (1-n, 12-w, 19-n, 7-w, 13w), but genetic analysis showed the sample clustered with other *L. cinereus* samples in lineage 23.

Reaction pattern 4 was identified in 12 of 14 RABV from *L. intermedius*. The reaction pattern of the two exceptional *L. intermedius* samples was not known to be associated with any bat species. Because the CDC repository contains only Florida *L. intermedius* samples, seven Texas *L. intermedius* samples of reaction pattern 4 were

Table 1. Bat species submitted to the Texas Department of Health laboratory for rabies virus (RABV) testing (1996–2000)

Species	Total no. received (1996–2000)	No. testing positive for RABV (%)
Antrozous pallidus (pallid bat)	3	0
Desmodus rotundus (vampire bat)[a]	4	0
Eptesicus fuscus (big brown bat)	14	1 (7.1%)
Lasiurus borealis (eastern red bat)	714	48 (6.7%)
L. cinereus (hoary bat)	57	15 (26.3%)
L. ega (southern yellow bat)	80	2 (2.5%)
L. intermedius (northern yellow bat)	153	14 (9.2%)
Lasionycteris noctivagans (silver-haired bat)	5	0
Lasiurus seminolus (seminole bat)	14	2 (14.3%)
Mormoops megalophylla (ghost-faced bat)	1	0
Myotis austroriparius (southeastern myotis)	1	0
M. californicus (California myotis)	1	0
M. ciliolabrum (western small-footed myotis)	1	0
M. thysanodes (fringed myotis)	1	0
M. velifer (cave myotis)	172	4 (2.3%)
M. yumanensis (yuma myotis)	1	0
N. humeralis (evening bat)	410	3 (0.7%)
Nyctinomops macrotis (big free-tailed bat)	5	0
Pipistrellus subflavus (eastern pipistrelle)	40	0
Tadarida brasiliensis (Brazilian free-tailed bat)	2,062	338 (16.4%)
Fruit bats, not speciated[b]	2	0
Juvenile yellow bats (*L. ega* or *L. intermedius*)	65	0
Lasiurus sp.[c]	24	1 (4.2%)
Unable to identify species[d]	159	6 (3.8%)
Total	3,989	434 (11%)

[a]Vampire bats part of captive colony in zoo.
[b]Appear to be two different species.
[c]Too damaged to determine species; will probably fall within one of the above mentioned *Lasiurus* sp.
[d]Extremely damaged, decomposed, or immature.

Table 2. Monoclonal antibody (MAb) reaction patterns of bat rabies virus variants (RABVV), Texas

Pattern	MAb 1	MAb 12	MAb 19	MAb 7	MAb 13	Bat species associated with RABVV
1	P	P	N	P	P	*Tadarida brasiliensis* (Brazilian free-tailed bat)
2	N	N	N	P	P	*Lasiurus borealis* (eastern red bat)
3	N	W	N	W	P	*L. cinereus* (hoary bat)
4	N	N	N	P	N	*L. intermedius* (northern yellow bat)
5	N	P	P	P	P	*Eptesicus fuscus pallidus* (big brown bat)

[a]P, positive; N, negative; W, weakly positive.

submitted for genetic analysis. Five of the seven samples clustered in lineage 29, a lineage new to the CDC repository and not in GenBank. Two of the seven samples clustered in lineage 28 with *L. intermedius* samples from Florida. The two Texas *L. intermedius* samples with unrecognizable MAb patterns (1-w, 12-n, 19-n, 7-w, 13-n; 1n, 12-w, 19-n, 7-w, 13-p, respectively) clustered in lineage 29 (txli5768) and lineage 23 (txli4260) by genetic analysis.

The single RABV sample from *E. fuscus* displayed reaction pattern 5. This sample was unavailable for genetic analysis, but a 1994 Texas *E. fuscus* sample (txef4250) with reaction pattern 5 RABV shared 99% identity with RABV samples from western big brown bats in lineage 1 (shown as representative samples from Colorado and Arizona).

The remaining 11 rabies-positive samples from Texas bats were collected from *M. velifer*, *L. seminolus*, *L. ega*, or *N. humeralis.* Because no MAb reaction patterns or genetic lineages have been established for these species, 10 of 11 samples were typed by antigenic and genetic methods. The MAb reaction pattern for one sample from *N. humeralis* was determined, but the sample was unavailable for genetic analysis.

The four samples from *M. velifer* displayed three different MAb reaction patterns. Sample txmv4267 displayed reaction pattern 1 and also clustered with other samples from *T. brasiliensis* in lineage 31 in the genetic analysis. The MAb reaction pattern of sample txmv4270 is not known to be associated with any bat species (1-n, 12-n, 19-n, 7n, 13-p), but the genetic analysis showed an association with *L. cinereus* in lineage 23. MAb reaction pattern 2 was found in txmv4258; however, genetic typing indicated lineage 5, a lineage new to the CDC repository. Lineage 5 was also found in sample txmv4256, which had displayed an MAb reaction pattern not known to be associated with any bat species (1-n, 12-p, 19-n, 7-w, 13-p). No other samples of lineage 5 exist in the CDC repository, and the repository contains only one other sample from *M. velifer* (from California). The California *M. velifer* sample clustered with *T. brasiliensis* samples in lineage 1 (not shown).

The *N. humeralis* samples (n = 3) had previously unrecognized yet identical reaction patterns (1-n, 12-p, 19-n, 7-p, 13-n), as did two reference samples from this species collected in 1995. Two of the 1998 *N. humeralis* samples (txnh4267 and txnh4269) and two reference samples (txnh3011 and txnh3012) indicated lineage 9, a lineage new to the CDC repository; that repository contains only one other sample of a lineage 9 RABV, an *M. austroriparius* from Florida (not shown). The only additional RABV sample from *N. humeralis* in the repository, also from Florida, clustered with *L. borealis* samples in lineage 26 (not shown).

The two RABV samples from *L. seminolus* displayed MAb pattern 2, associated with *L. borealis*. Both samples (txls4274 and txls5769) clustered with RABV from *L. borealis* in lineage 26. Three additional samples from *L. seminolus* in the CDC repository (all from Florida) also clustered with *L. borealis* samples (not shown).

The *L. ega* sample (txle4266) displayed a unique MAb pattern (1-p, 12-n, 19-n, 7p, 13-p) and clustered in lineage 22 with three samples from *L. ega* bats from Arizona. Four additional samples from *L. ega* in the CDC repository did not contain a lineage 22 RABV. These samples contained lineages 1 and 23, which suggests infection through contact with *T. brasiliensis* and *L. cinereus*, respectively.

Conclusions

For those laboratories without genetic typing capability, antigenic analysis with MAbs offers a rapid, simple, and inexpensive means of typing RABV for epidemiologic surveys. Our study suggests MAb typing can be useful for large-scale surveys in which hundreds to thousands of virus samples originate from only one or two bat species and the question is simply "Do we find in these species the RABVV that we expect to find?" All but 5 of 407 samples from *T. brasiliensis*, *L. borealis*, *L. cinereus*, *L. intermedius*, and *E. fuscus* tested in this study displayed the MAb patterns expected for the species. However, MAb typing by fluorescence microscopy lacks precision. Surveys that rely solely on antigenic typing underestimate the true diversity of RABV in bat populations and may oversimplify rabies transmission cycles. For example, antigenically identical samples from both *L. borealis* and *L. intermedius* segregate as different genetic lineages (Figure) (13). This pattern of divergence does not correlate with time or the area in which either species was collected and must reflect some as-yet unknown aspect of natural history that partitions and segregates virus populations. These findings suggest not only that genetic typing offers

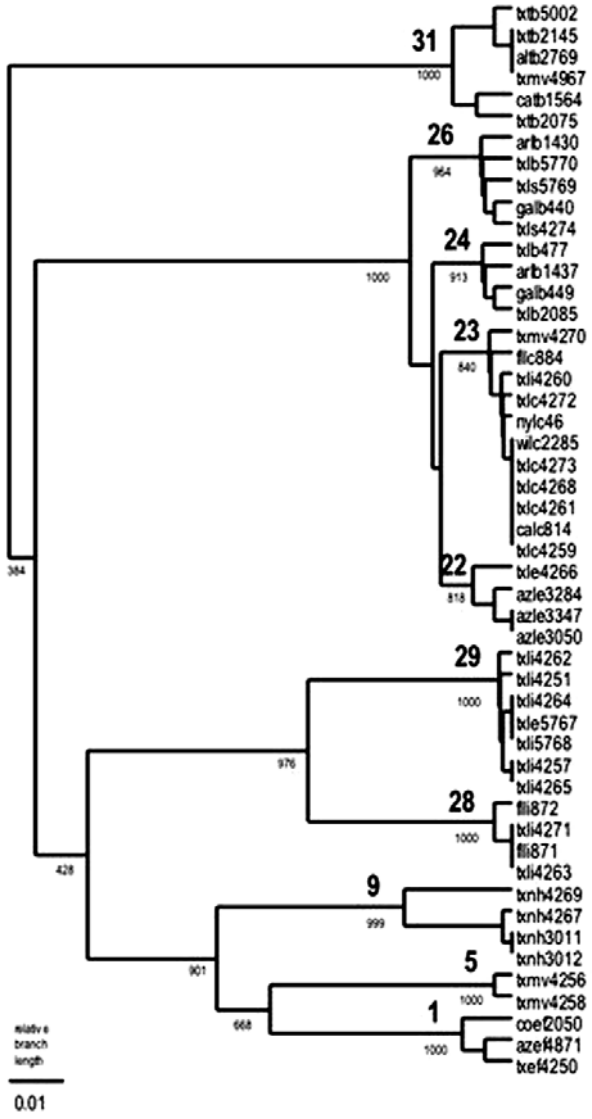

Figure. Genetic lineages of rabies virus in Texas bat populations. Lineages were determined by using the unweighted pair group method using arithmetic averages (UPGMA) method on the basis of 302 nucleotides of nucleoprotein gene sequence. Bootstrap values of 1,000 replicates indicate the robustness of the corresponding node. Representatives of each lineage were deposited in GenBank under the following accession numbers: 31, AF394876; 26, AY039224; 24, AF394886; 23, AF394883; 22, AY170247; 29, AY208163; 28, AF394878; 9, AY208164; 5, AY208165; 1, AF394887.

a more precise identification of a RABVV but also that genetic analysis of RABV may help us better understand how the natural history of the host drives viral evolution.

The observed genetic diversity among the 23 samples sequenced for this study was unexpectedly large for such a small sample set. Two lineages (5 and 29) consisted solely of Texas samples; lineage 9 had been identified previously in only one other sample (an *M. austroriparius* from Florida); and lineage 22 had been identified previously only in *L. ega* samples from Arizona. The small number of samples in these four lineages does not allow designation of reservoir status for these species, but the genetic diversity in the RABV in Texas reflects the diversity of bat species in the southwestern United States and suggests that many, if not all, bat species transmit distinctive RABV. Identification of the species association of different variants of RABV could lead to valuable information about routes of virus transmission and mechanisms by which RABV persists in different bat populations.

Acknowledgments

We thank Letha Zuckero and Diane Preusse for assistance with bat identification; Pushker Raj for his support of the project; Pam Wilson, Bradley Hicks, and Guy Moore for editorial advice and bat identification assistance; and Barbara French and Cullen Geiselman for bat identification and geographic distribution information.

This work was supported by the Texas Department of Health and by a Research Enhancement Grant from Texas State University at San Marcos.

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Human and Porcine Hepatitis E Virus Strains, United Kingdom

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We describe a case of acquired infection of a strain of hepatitis E virus (HEV) with a 100% amino acid identity to the analogous region in strains of HEV circulating in a United Kingdom pig herd. This case further supports the theory that autochthonous HEV infection in industrialized countries is zoonotic.

Hepatitis E virus (HEV) is a common cause of acute hepatitis in many developing countries (1). HEV is often waterborne and causes an illness similar to hepatitis A. During the icteric phase, patients may be viremic (2). In the last few years, sporadic cases of hepatitis E have been reported in industrialized countries in the absence of foreign travel or other known risk factors. Nucleotide sequence analysis of some of these viruses has indicated a high degree of sequence identity with the sequences of HEV detected in pigs (3–5) and in pig meat purchased in retail outlets in Japan (6). More recently, the most direct evidence of zoonotic transmission yet available was described in a case study from Japan. In this case, members of a family group were infected following consumption of uncooked Sika deer meat. The virus identified from the patients was shown to be identical to that recovered from uneaten quantities of meat from the same deer (7). In the United Kingdom, autochthonously acquired hepatitis E has been reported rarely and a short (98-bp) section of the virus genome has been sequenced from one such patient (8). Serologic evidence of HEV infection has been detected in U.K. pigs in a preliminary study (9) In two pigs from separate farms, an HEV genome was detected by reverse transcription–polymerase chain reaction (RT-PCR) and sequenced (strains 1-40 and 14-P354). These sequences identified the porcine strains as nearly identical to the 98-bp U.K. human HEV sequence in HEV genotype III (9). Further study is underway to determine the prevalence and strain diversity of HEV infection in U.K. pigs.

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The Study

In June 2000, a 58-year-old woman, who worked as a shop assistant, was seen at a rapid access "jaundice hotline" clinic with a 5-day history of myalgia and jaundice. She had not traveled outside the United Kingdom for 10 years and had no contact with farm or domestic animals. She was not a vegetarian and, although she admitted to eating raw sausage and bacon in the past, she claimed not to have done so in the 3 months before her illness. Examination confirmed jaundice and tenderness in the right upper quadrant. Her liver function tests showed elevated levels of bilirubin, 46 μmol/L; alanine aminotransferase, 2,421 IU/L; and alkaline phosphatase, 200 IU/L. Blood drawn at this time was positive for anti-HEV immunoglobulin (Ig) M; no serologic evidence indicated active infection with Epstein-Barr virus, cytomegalovirus, or hepatitis A, B, or C. Results of a liver ultrasound were normal, and serologic evidence did not indicate autoimmune or metabolic liver disease. One month later, she felt better, was no longer jaundiced, and results of her liver function tests were generally normal. A diagnosis of acute hepatitis E was made.

Total RNA was extracted in Trizol (Sigma, Poole, UK) from a blood sample drawn from the patient at the time she sought treatment. Template cDNA from the blood extraction was prepared by a reverse transcriptase step, according to standard protocols. A nested PCR was used to detect HEV from the prepared cDNA samples. This assay used degenerate oligonucleotide primers to amplify a 348-bp fragment of open reading frame 2 (ORF2) of HEV as described by Meng et al. (1997) (10).

Cycling parameters were the same as those described by Meng et al. (10), except for annealing temperatures of 60°C and 55°C for external and internal primer pairs, respectively, and an extension time of 1 min. RT-PCR products were subjected to electrophoresis on a 2% agarose gel containing ethidium bromide and visualized under UV light.

Amplicons of correct size were excised from 2% agarose gels, purified with QIAquick Gel Extraction Kit (Qiagen Ltd., Crawley, UK), and sequenced with Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Warrington, UK). Sequences comprising 304 bp of HEV ORF2 were assembled by SEQMAN (DNASTAR) and phylogenetic analysis was performed using PHYLIP (ver 3.6) with NEIGHBOR. Bootstrap confidence values were calculated by using SEQBOOT and CONSENSE (available from http://evolution.genetics.washington.edu/phylip.html).

Analysis of the 304-bp nucleotide sequence obtained between the primer sequences showed 16 and 32 nucleotide differences from the two U.K. pig strains 1-40 and 14-P354, respectively. However, all of these differ-

ences were silent when the gene sequence was translated into amino acids; the putative amino acid sequence of the virus detected in the patient had 100% identity with that of the two U.K. pig strains. Based on the nucleotide data the phylogeny of the three strains was determined (Figures 1 and 2), showing a very close relationship between the three strains in the genotype III lineage. In Japan, high sequence similarities have been demonstrated between human and pig HEV strains within genotype III and genotype IV (5).

Conclusions

HEV was discovered in pigs as recently as 1997 (10), despite evidence from several countries that it had been present since at least the early 1990s. The lack of recognized clinical disease in pigs (11) was undoubtedly a factor in this late discovery. The similarity of the human strain detected in this case to the two U.K. pig strains is suggestive of zoonotic transmission, as is the finding of higher prevalence of anti-HEV antibodies in those who work with pigs (12) and the observation that in 9 of 10 hepatitis E patients in Japan, the disease developed within 2–8 weeks of consumption of grilled or undercooked pork (6). Consideration is being given within the U.K. Department for Environment, Food and Rural Affairs to research proposals to gain better understanding of HEV in the U.K. pig herd. Screening patients who present with acute hepatitis for evidence of HEV infection is a simple way to improve the assessment of human HEV infection. Rapid access jaundice hotline clinics (13) are an ideal venue for such testing because patients may present while still viremic, allowing molecular characterization of infecting viruses as in this case. Work is currently in progress to assess the incidence of hepatitis E in a cohort of over 650 acutely jaundiced patients who have contacted the jaundice hotline in the past 5 years.

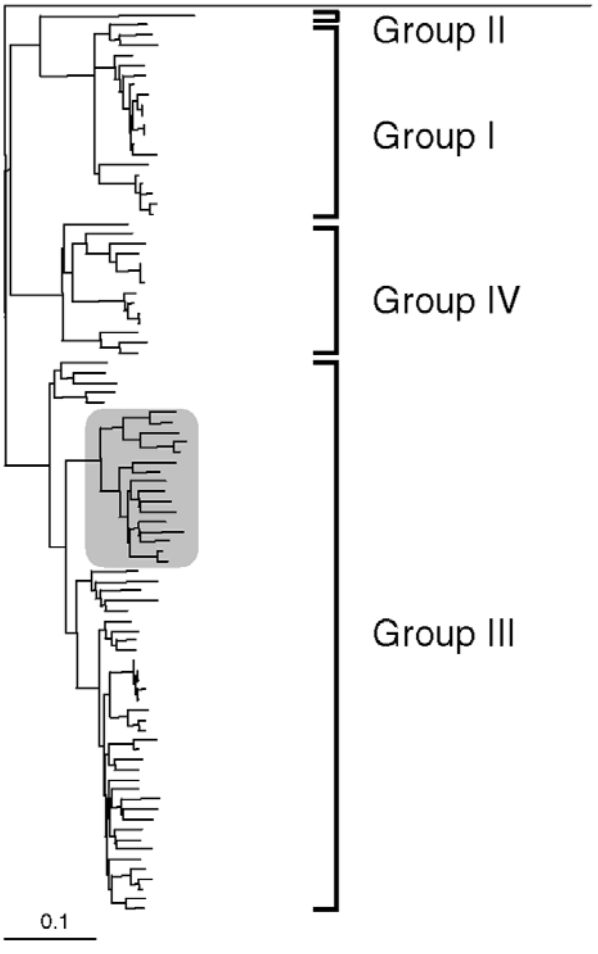

Figure 1. Outline of dendrogram of selected partial nucleotide sequences of ORF-2 region of swine and human hepatitis E virus (HEV) isolates (300 nt). Avian hepatitis E virus (AY043166) was chosen as an outgroup for these analyses. Genotypic groupings are indicated. Clustering of the human UK HEV isolate and closely related sequences is highlighted within shaded area (Figure 2).

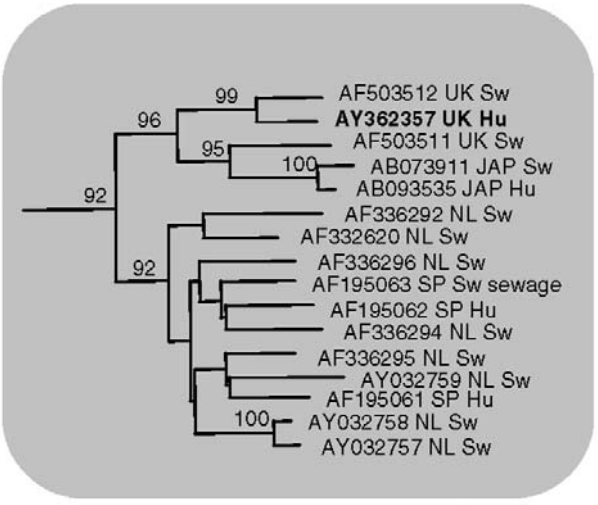

Figure 2. Human United Kingdom isolate (AY362357) is shown in bold and compared with closely related swine and human hepatitis E virus isolates (GenBank accession no., country of origin, and host are indicated). Bootstrap values greater than 70% are considered significant and are indicated.

This work was supported in part by Defra project SE0515

Dr. Banks has been a research virologist at the Veterinary Laboratories Agency for over 20 years. He has a background in the control of animal herpesviruses, working with the European Union and Office Internationale des Épizooties. His current research interests lie in the pathogenesis of porcine multisystemic wasting syndrome and the incidence and prevalence of animal hepatitis E virus in relation to zoonoses.

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Setu Vora, photographer

Medical students on community health visit to the remote villages of Gujarat, India.

Living with the Plagues

Setu K. Vora*

A series of massive explosions rocked Mumbai, India, on March 12, 1993, killing at least 200 and injuring thousands. Shock, agony, anger, and fear spread from the clouds of smoke and debris. Then came corrosive anxiety—the hallmark of our age. Belief in fate and karma kept us going as we learned to live and cope with terrorism. Then, just as monsoon follows Indian summer, another tragedy came. In September, an earthquake near Latur, Maharashtra, flattened villages, killed thousands, and spawned bubonic plague. We drowned our sorrows in festivals, cricket matches, and movies, and we lived on.

I was in medical school when the next disaster struck in 1994. They came with their faces covered with handkerchiefs. Luggage in hand, they rushed from buses or trucks outside the hospital into the emergency room, where I was an intern. The first few masked patients did not attract much notice, but a roomful of them clamoring for attention and begging for tetracycline created a ruckus. Torrential rains had flooded the city of Surat on the bank of the river Tapti, and mystery pneumonia gripped the population, killing them quickly with respiratory failure. The word "plague" was all over the streets and the news. The local doctors were among the first to flee. "Diamond City" lost its luster as immigrant workers scrambled on to trains, buses, and trucks, toward their hometowns. Surat was desolate. Flights and exports from India were banned, garbage and rats ruled the streets, and panic settled in. Then, the floodwaters of the river receded, and the outbreak subsided as quickly and mysteriously as it had erupted.

The reemergence of Black Death caused much soul-searching among the intelligentsia. They blamed it on the earthquake in Maharashtra, monsoon floods, poor sanitation, and angry gods. "Foreign hand" alarmists raised the specter of bioterrorism. According to the Hindustan Times, a highly placed official, on condition of anonymity, said, "We already know that the Surat strain was not Indian. We cannot rule out the possibility of militants purchasing the organisms from a Kazakhastan company and releasing them in Surat." Somehow, believing a disease is exotic and imported is comforting. This outbreak left 56 dead; malaria and tuberculosis, which kill thousands daily, do not generate half as much excitement or introspection—with familiarity comes tolerance. This was my first encounter with the plague.

Three years later, an internal medicine resident and first responder to a disease outbreak, I did not know if I should be proud or petrified. This responsibility was not mentioned in the residency curriculum; in fact, I do not recall seeing a curriculum. In the rain-drenched, fertile farms of southern Gujarat, sugarcane and paddy farmers were reaping more than they sowed. They were coming down with high fever, severe body pains, jaundice, and kidney failure. Leptospirosis was raging, and I was sent to care for patients at Navsari Civil Hospital. The train ride from Baroda to Navsari was my chance to learn about leptospirosis. At the hospital, I was escorted to my room adjacent to the leptospirosis ward. I was relieved to see a

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mosquito net over my bed. I was well-prepared to face Weil syndrome. After all, I had observed insertion of one peritoneal dialysis catheter and had even inserted one myself before I reached Navsari. I may have saved a few patients from uremic deaths, and many that died in spite of my efforts probably would have died of the disease anyway. I maintained a database of 71 cases, logging patients' initial symptoms, laboratory tests, and clinical course. A few likely had other diseases, malaria or dengue fever, but we had no way of knowing right away, since sera were sent to New Delhi for testing. I never saw the results. Back in the medical college, professors were pleased with the media coverage of their generously lending resident doctors to a noble cause. My 2-week tour of duty was soon over. One month later, cerebral malaria caused by *Plasmodium falciparum* was the new killer stalking villages, this time in the northern districts. I was relieved to be gone by that time.

I escaped these plagues by leaving India behind for the medical world of the United States. Physicians with an accent are accepted in the U.S. healthcare system, and patients are often curious to know more about them. Patients often ask me, "Where do you come from, India or Pakistan?" This is usually followed by polite remarks, "I have always wanted to visit India," or "I love Indian food." I never know how to respond to the question, "What made you come to the United States?" The usual answer is, "To learn advanced medicine and research." But I know there is more to it. I love India dearly, but I did not have the courage or the patience to deal with her unmet potential, challenges, and misplaced priorities.

I admire and envy a friend who went back to India after a brief stint at Johns Hopkins. He and other like-minded physicians now work in rural India, combating malaria, malnutrition, and maternal and infant deaths. They struggle against apathy, politics, and poverty. Like Sisyphus, they push TB and AIDS up the hill each day, only to see them roll back again each night. I chose to practice healing in a setting free of mosquitoes, politicians, charlatans, and terrorists.

When mosquito-borne West Nile virus encephalitis emerged in New York in 1999 and rapidly spread throughout the United States, it brought with it the familiar insecticide spraying and fogging, reminding me of my old home. Once again, bioterrorism was the whispered cause of this emerging disease. Then the events of September 11, 2001, scarred my adopted homeland forever. My wife saw the airplanes plunge into the towers from her safe vantage point on the West Side. The plague of terrorism had arrived, soon followed by an intentional outbreak of anthrax, which changed forever the way we handle mail and look at white powder.

Not all infectious threats we face are of exotic origin. During my fellowship in critical care, the once-familiar bugs *P. vivax*, *Entamoeba histolytica*, and even *Salmonella* almost felt benign when compared to methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, and multidrug-resistant gram-negative bacteria that lurk in shiny hospitals in the United States. Our system valiantly supports many terminally ill patients shuttling between nursing home and intensive care unit for catheter or device-related infections. These patients and their next of kin still have the right and luxury to crave the elusive cure. Within months, these patients become petri dishes, reflecting all the prevalent nosocomial pathogens in a hospital. Superbugs are also born in the United States.

The plagues may have some evolutionary role or may be designed to save us from our hubris. As predicted by Hans Zinsser, "…however secure and well-regulated civilized life may become, bacteria, protozoa, viruses, infected fleas, lice, ticks, mosquitoes, and bedbugs will always lurk in the shadows ready to pounce when neglect, poverty, famine, or war lets down the defenses" (Rats, Lice, and History; 1934). Eradicating the plagues seems futile if we do not address the factors that trigger them.

Disasters followed by plagues recur throughout the world. An earthquake in India that spawns plague, ecologic disasters in Africa that unleash Ebola, and terrorism in the United States piggybacked by anthrax make all nations vulnerable. Poverty, ignorance, hatred, and infection can breed in lands across the ocean and reach us by air to poison our existence. As a physician, I could not escape the plagues by moving away. I could only exchange them for new ones. According to the ancient Sanskrit concept of *Vasudaiva Kutumbakam*, the world is one family. We are likely to eat contaminated imported food and travel to and host visitors from hot zones of disease and terror. Heightened surveillance for imported disease may not be sustainable unless wedded to primary prevention and control of disease at its source. The plagues know no boundaries, and in our efforts to prevent them, neither should we.

Dr. Vora is currently a fellow in Pulmonary and Critical Care Medicine with special interest in pulmonary infections, especially after transplantation, and biologic therapies for rheumatologic diseases. He is also interested in intensive care unit preparedness against bioterrorism or emerging infectious diseases. Outside of work, he enjoys investigative medical humanities, poetry, and photography.

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Beijing/W *Mycobacterium tuberculosis* in Italy

To the Editor: Molecular typing of *Mycobacterium tuberculosis* strains isolated in several countries in recent years has shown that a group of strains known as "Beijing" is widespread around the world (1). The Beijing group of *M. tuberculosis* has been associated with drug resistance; one multidrug-resistant strain, designated "W," was found in New York City in the early 1990s and caused large institutional outbreaks of tuberculosis (TB) in the United States (2). *M. tuberculosis* strains of Beijing/W genotype are mostly prevalent in Asia (1), but recent data suggest that they have been spreading in Indochina and are prevalent among younger persons in Vietnam (3). Beijing/W strains are also widespread in Eastern Europe (1); during the last decade, the Beijing/W genotype of *M. tuberculosis*, with more prevalent drug-resistant mutations than non-Beijing strains, has been identified in 40% to 50% of clinical isolates studied in Russia (4).

We studied a total of 245 *M. tuberculosis* strains collected during a 1-year period, from January to December 2002, from the same number of TB patients hospitalized in Tuscany, Italy. All the isolates were typed by the standardized IS*6110* restriction fragment length polymorphism (RFLP) and the spoligotyping (spacer oligonucleotide typing) techniques. A total of 216 distinct IS*6110* RFLP patterns were found among the 245 isolates; 51 isolates (20.8%) occurred in 23 clusters, each constituting strains with an identical IS*6110* RFLP and spoligotype pattern; 19 clusters contained two isolates each, 3 contained three isolates, and 1 contained four isolates. Spoligotype analysis showed seven isolates with the typical Beijing/W pattern of probe hybridization only to spacer sequences 35–43. The Beijing/W isolates yielded distinct IS*6110* RFLP profiles with similarity coefficient >57.8%. Characteristics of the Beijing/W strains and respective patients, obtained from clinical records, are reported in the Table. Although the overall prevalence of Beijing/W strains was low (7/245, 2.9%), five of the seven strains were from recent immigrants to Italy from China who live in the same area; the other two strains were from Italian citizens also living in that area. Recent immigration from high-prevalence areas is therefore likely to be associated with the occurrence of the Beijing/W genotype in Italy. None of the Beijing/W strains was associated with TB outbreaks; nonetheless, infection of Italian residents with Beijing strains suggests that spread of this genotype is ongoing.

Beijing/W strains have been strongly associated with drug resistance in a number of countries (2,4–6), but elsewhere the association was weak or absent. In our survey, no substantial drug resistance was observed; all Beijing/W strains isolated in Tuscany were susceptible to rifampin, ethambutol, pirazinamide, and streptomycin (tested only in two strains), and all but one were susceptible to isoniazid.

Although we detected only a few cases, our data do not show a trend of Beijing/W strains' being associated with infection in young people, as has been observed in other settings (3). The age of immigrants with Beijing/W TB (mean 33.2 years, standard deviation [SD] 8.2 years) did not significantly differ from that of immigrants infected with non-Beijing/W strains (30.7 years, SD 7.4 years), a find that indicates that, at least in our setting, immigrant status, rather than *M. tuberculosis* genotype, is associated with infection in young people. The few cases of Beijing/W infections in Italian-born patients do not allow us to draw conclusions regarding non-immigrant patients.

In conclusion, *M. tuberculosis* strains of Beijing/W genotype are becoming widespread worldwide, including in countries with a low prevalence of TB. Their association with drug resistance and infection in young people, clearly shown in certain settings, remains to be defined. Further molecular epidemiologic surveillance is needed to monitor trends in prevalence and spread of these strains.

Table. Characteristics of *Mycobacterium tuberculosis* strains of Beijing/W genotype isolated in 2002 in Tuscany, Italy[a]

Strain no.	Patient's country of birth	Sex	Age	HIV status	Years in Italy	Site of TB	Drug resistance[b]				
							Str	Inh	Rif	Eth	Pza
669	China	M	40	–	1	Pulmonary	S	S	S	S	S
763	China	M	42	–	<1	Pulmonary	S	S	S	S	S
804	China	F	23	–	4	Pulmonary	NT	S	S	S	S
836	China	M	34	–	1	Pulmonary	NT	S	S	S	S
884	Italy	F	39	+	NA	Extrapulmonary	NT	S	S	S	S
952	Italy	F	28	–	NA	Pulmonary	NT	R	S	S	S
974	China	F	27	–	1	Pulmonary	NT	S	S	S	S

[a]TB, tuberculosis; Str, streptomycin; Inh, isoniazid; Rif, rifampin; Eth, ethambutol; Pza, pirazinamide; S, susceptible; R, resistant; M, male; F, female; NA, not applicable; NT, not tested.
[b]Drug resistance was assessed by the radiometric BACTEC system (Becton Dickinson, Towson, MD) according to the proportion method.

This work was supported by National Research Program on AIDS grant no. 50D.11 from the Istituto Superiore di Sanità, Rome, Italy.

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Animal-to-Human SARS-associated Coronavirus Transmission?

To the Editor: Martina et al. reported that domestic cats and ferrets are susceptible to infection by severe acute respiratory syndrome (SARS)–associated coronavirus (SARS-CoV) isolated from a patient infected with SARS. These infected animals could efficiently transmit the virus to uninfected animals housed with them (1). This finding is similar to that of SARS transmission in humans in which SARS-CoV can be quickly spread from person to person through close contact. Ferrets and domestic cats not only can be infected by SARS-CoV in the laboratory, but also can shed SARS-CoV from the pharynx at 2 days postinfection and continuing through 10 and 14 days postinfection, respectively (1). No clinical signs were observed in six cats that were injected with SARS-CoV, whereas three of six ferrets that were injected with SARS-CoV became lethargic within 2 to 4 days postinfection, and one of the three ferrets died at day 4 postinfection (1,2). This finding indicates that domestic cats may not only be a useful animal model for evaluating candidate vaccines and drugs against SARS (1) but also may be good reservoirs of SARS-CoV. Domestic cats living in the Amoy Gardens in Hong Kong, where >100 residents contracted SARS in the spring of 2003, were infected with SARS-CoV (1,3). This fact suggests that domestic cats can be naturally infected with SARS-CoV from humans infected with SARS, although how this SARS-CoV transmission occurs is unclear. Unfortunately, however, the transmission capability of the SARS-CoV strain transmitting from domestic animal to human, despite the widely accepted hypothesis of the animal origin of SARS-CoV (4–6), cannot be ascertained. If the transmission of SARS-CoV from animal to human is as easy as that from humans to domestic cats, the speculation that the outbreak of SARS in the Amoy Garden in Hong Kong was caused by environmental sources, such as U-traps in bathrooms contaminated with SARS-CoV (3), we should reevaluate, because this outbreak of SARS in these apartments might also be caused by infected cats or other mammalian hosts.

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The 1947 Smallpox Vaccination Campaign in New York City, Revisited

To the Editor: In 1947, millions of New Yorkers received smallpox vaccinations, an accomplishment still appropriately held up as an example of public health planning and mobilization. Although now mythological, a review of the events of April 1947, from copies of The New York Times (1–9), tells of a more recognizably human response: pushing, jawing, deceit, shortages, surpluses, and perhaps a unusual way of counting vaccinees.

In March 1947, a patient who had recently visited Mexico traveled by bus to New York City. He became ill, was hospitalized, and, after his death, found to have had smallpox. The occasional case of smallpox had been seen in the area for decades since the last big outbreak in 1875, which had killed 2,000 New Yorkers. However, in 1947, a second case and then a third appeared, and authorities became concerned. On April 4, Israel Weinstein, the New York City Health Commissioner, urged all New Yorkers who had not been vaccinated since childhood to receive another vaccination.

The program worked at the outset. Free vaccine clinics were established throughout the city, and doses were given to private physicians for administration. During the first week, surprisingly little public attention was captured (Times articles typically were brief and confined to page 21). The story hit page 1 on April 13 (3), after a second person died from the disease. Mayor William O'Dwyer urged all 7.8 million New York residents to receive the vaccine. Then he rolled up his sleeve and was vaccinated by Dr. Weinstein. The city swiftly swung into full crisis mode. Police, fire, and health departments and hospitals were mobilized to provide additional space for the effort.

Two days later, epidemiologic investigation indicated that all patients with diagnosed cases were related and that, in all likelihood, the outbreak had been successfully halted through tracing the movements of the various patients and vaccinating anyone who had contact with them, so-called "ring" vaccination (4). Despite this halt of the outbreak, the city pushed forward. The campaign to "Be sure, be safe, get vaccinated!" had proven successful. By city estimate, >600,000 persons had received vaccine in the first week.

Vaccine side effects, which dominate coverage of today's vaccination program, were seldom discussed in 1947. Dr. Weinstein assured residents, "Vaccination is painless. The skin is not even broken by the needle. Sometimes a soreness develops in the armpit. If the arm becomes very sore, apply an icebag" (4). This advice is simple compared to the depth and breadth of information given today to a potential vaccinee. Now, volunteers are given several informational lectures and a protracted individual interview to discuss lingering questions, and they are required to sign a document confirming adequate comprehension and acceptance of the risks.

In the 1947 campaign, trouble began on April 16, when (no longer on page 1), the Times announced, "Vaccinations Stop; Drug Supply Gone; Thousands Turned Away" (5). With little warning, and at the height of the program, the vaccine supply vanished, something that was never explained. After spending days gearing up citizens to receive the vaccine quickly, the mayor and Dr. Weinstein now had to downplay the urgency of receiving vaccination. They assured New Yorkers that a delay of a few days or more represented "no health hazard" (5).

Of the 1.2 million doses distributed by April 16, 1947, 42,000 had been supplied by private laboratories, far short of the promised number. In contrast, the Army and Navy had given almost 800,000 doses, and the city's public health laboratories had made the remaining 400,000.

During the shortage, the Times noted, "hundreds of eager men, women, and children queued up at Bellevue Hospital at dawn, although vaccinations were not scheduled to begin until 10 a.m. At some stations, the crowds did not take kindly to the news that the doctors had run out of vaccine and the police had a little difficulty dispersing a crowd of several hundred" outside one vaccine station (5).

On April 17, the situation brightened, when more than a million doses suddenly arrived from private laboratories, and 500,000 persons were vaccinated (6). As the crisis slowly lessened, doctors were recruited at US$8 (US$64 in today's market) for a 3-hour session (or US$24 for all day; US$192 in today's market) to administer vaccine, but few volunteered. Public health authorities in Westchester County chided local physicians for charging $35 per vaccine (7), and a 29-year-old woman, dressed up as a nurse, vaccinated 500 people with water to impress her "man companion" until she was sent to the Bellevue psychiatric ward for evaluation (8).

Continued complaints about side effects were dismissed by Dr. Weinstein, who again advised those whose arm ached that they only needed to place an icebag in the armpit for relief. Within a week, the program had wound down and been proclaimed "a miracle" (2) by all involved.

The claim of 5 or 6 million vaccinations administered cannot be reconciled against the daily tally reported in the Times. If one assumes that day-to-day numbers reported in the newspaper were roughly accurate, a simple calculation places the number of vaccinees closer to 2.5 million, far short of the announced total. For example, on April 21, a grand total of 3.45 mil-

lion recipients were reported; the next day, after noting that only 200,000 additional persons had received vaccine, the total swelled to 4.4 million (9).

These data reflect the difficulties intrinsic to managing such a massive program. The discrepancy may simply be a case of not adding columns of numbers in a systematic way; however, the fuzzy numbers do have a certain appeal to the modern, more cynical reader.

Whatever occurred, understanding the specifics of "the great vaccination miracle" of 1947 is important for maintaining equilibrium during our current smallpox vaccination program and any future programs directed at now-unanticipated infections. Not just New York City's, but the entire country's sense of confidence that it can handle a major rapid vaccination or pill distribution campaign leans very heavily on the apocryphal vaccine campaign of April 1947. Yet, as described above, there may be much less to the miracle than meets the eye.

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Smallpox Vaccination and Adverse Cardiac Events

To the Editor: The incidence of adverse cardiac events related to smallpox vaccinations administered during the National Smallpox Vaccination Program (NSVP) in 2003 has received widespread attention. From January 24 through August 8, 2003, suspected or probable myo- or pericarditis was reported in 22 of 38,257 civilian vaccinees (1); as of November 4, 2003, suspected or probable myo- or pericarditis was reported in 63 of 515,000 military vaccinees (2). Additionally, cases of coronary artery disease, including myocardial infarction and cardiac death, were reported in the weeks after vaccination although no causal link has been established.

An October 3, 2003, MMWR article, "Cardiac deaths after a mass smallpox vaccination campaign—New York City, 1947" states that the NYC experience suggests "…that cardiac deaths observed in 2003 might have been unrelated to smallpox vaccination." While the causes of these cardiac or coronary deaths have not been established, the 1947 data lack the power to address whether there is a relationship to the vaccine.

Cardiac or coronary deaths after vaccination in 2003 were rare, with a total of 3 of 488,550 military and civilian vaccinees (6 per 1 million vaccinees), approximately the same as might be expected in a generally healthy population. The total number of cardiac or coronary deaths in 1947 during the 2-week estimated risk period after vaccination was 1,545. While the denominator (number vaccinated in the previous 4–17 days) was not reported, a total of 6.4 million persons were vaccinated during the 4 weeks of the vaccination program. The 4-week vaccination period would result in a 6-week period of susceptibility for cardiac death according to the 4–17 day latency period. Thus, we extrapolate that the denominator for the 2-week observation period is approximately 2.1 to 6.4 million vaccinees at risk during the study period. This would mean that approximately 240 to 720 cardiac deaths occurred per million vaccinees.

Suppose that the 1947 smallpox vaccine indeed caused serious cardiac disease, including myopericarditis and myocardial infarctions, with 10 fatal cases per million. Viewed in perspective, this would approximate the historic rate of vaccine-induced encephalitis and would be well in excess of the historic rate of progressive vaccinia. In this scenario, at a hypothetical incidence of 10 per million, from 21 to 64 of the 1,545 cardiac deaths (1.4% to 4.1%, respectively) would have been caused by the vaccine. This magnitude of effect would have been very difficult to detect in this study. Thus, the results of such investigations must be considered in the context of power limitations. Further, studying death rates sheds no light on cardiac illness such as myo- or pericarditis.

The proper interpretation of these data is important given the national policy impact that resulted from the observation of cardiac and coronary illness and death after vaccination in 2003. At this time, adverse cardiac events associated with the vaccine, particularly myo- or pericarditis, are still of concern. Whether coronary or cardiac deaths can be attributed to the vaccine remains an open question.

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In Reply: We have reviewed the letter submitted by Upfal and his colleagues (1), and we applaud their careful examination of our previously published data (2). Their aim was to assess whether the study was adequately powered to detect a small but potentially relevant effect in cardiac death rates. This question is important, and their message regarding the difficulty of measuring small effects is certainly true. We address these problems in the discussion section of our article published in this edition of Emerging Infectious Diseases; however, we would also like to clarify some points that were misleading in their letter.

While the methods Upfal et al. employ to assess statistical power are generally correct, they base their argument on estimates derived from 2003 deaths in both the civilian and military population. We remind readers that the military is a unique group, more physically fit and potentially younger than the general population, today and in 1947. A more appropriate estimate of potential risk for the general population (i.e., what we would have seen in 1947 if a vaccine-associated risk for cardiac death existed) would be to use risk estimates derived from deaths among civilians. If observed civilian deaths in 2003 were indeed vaccine-associated, our study certainly had the power to detect such an effect in 1947.

Also, when calculating the rate of cardiac deaths among 2003 vaccinees, Upfal et al. refer to additional 2003 vaccinations in the military that have occurred since the time our article was published. Since no additional deaths occurred, these additional vaccinations further dilute the risk. However, this larger number of vaccinees modestly affects the estimated risk size. In the Table below, we show that our study had sufficient power to detect effects of a relevant size.

We agree with Upfal's basic premise that our study lacks the statistical power to detect very small risks (such as 1% increases), but most studies struggle with the same limitation. Our study does provide useful and convincing evidence that neither moderate nor large increases in cardiac mortality occurred in 1947 as a result of smallpox vaccination.

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Table. Smallpox vaccination and estimated risk for cardiac death

	All cardiac deaths[a]	Atherosclerotic deaths[a,b]	All cardiac deaths, civilians only[c]
Estimated vaccine-associated death risk (deaths/vaccinees) based on 2003 experience[d]	6.1 per million (3/488,550)	6.1 per million (3/488,550)	52.3 per million (2/38,257)
1947 rate of cardiac deaths in 2-week risk period (deaths/vaccinees)	241 per million (1,545/6.4 million)	44 per million (280/6.4 million)	44 per million (280/6.4 million)
Percent of 1947 deaths that would have been due to vaccination, given estimated vaccine-associated risk	2.5%	14.0%	100%
Sufficient power to detect?	Possibly	Definitely	Definitely

[a]For these estimates, we used the total number of vaccinees as of November 2003, per Upfal's letter. Our original article estimated risk based on 394,584 vaccinees as of April 2003; the resulting effect size would be even larger and more easily detectable.
[b]All cardiac deaths in 2003 were atherosclerotic.
[c]For this calculation, we used the total number of civilian vaccinees as of August 2003, per Upfal's letter. Our original article estimated risk based on 29,584 civilian vaccinees as of April 2003; the resulting effect size would be even larger and more easily detectable.
[d]Estimated risk for death caused by vaccine, if one assumes that all 2003 cardiac deaths were vaccine-related.

Bartonella Species Isolated from Rodents, Greece

To the Editor: Domestic cats and human body lice have been identified as the vectors of *Bartonella henselae* and *B. quintana*, respectively, the primary sources of *Bartonella*-associated human diseases (1). *Bartonella* species are zoonotic agents that have been isolated from a wide range of mammals in the United States (2) and Europe (3) and have been associated with human diseases (4–5).

This study investigated the potential for infection from *Bartonella* species in rodents in northern Greece. The small mammals tested were collected with live traps (6). Two sites were surveyed; the first was Nevrokopi, a small town in the Rhodope Mountains near the Greek-Bulgarian border, and the second site included Pramanta, a small village in the Pindos Mountains, and Matsuki, a small village in northwestern Greece. At Nevrokopi, 57 small mammals were captured during 887 trap nights for a success rate of 6.4%. At Pramanta and Matsuki, 13 small mammals were captured during 400 trap nights for a success rate of 3.3%. The 70 captured mammals comprised seven species of rodents. *Apodemus flavicollis* was the most commonly captured species (87%). Blood samples from each of the trapped mammals were frozen in liquid nitrogen in the field and subsequently stored at –70ºC before bacteria isolation. Bacteria isolation was performed as previously described (7). One hundred microliters of whole mammalian blood was cultured on heart infusion agar containing 5% rabbit blood (Becton Dickinson, Franklin Lakes, NJ) and incubated in 5% CO_2 at 35°C for a minimum of 4 weeks. DNA of the putative *Bartonella* cultures was extracted by using QIAamp Tissue Kit (Qiagen GmbH, Hilden, Germany). Polymerase chain reaction (PCR) was performed by using two oligonucleotides specific for the citrate synthase (*glt*A) gene of *B. henselae* Houston 1, primers BhCS 781.p and BhCS 1137.n. Negative and positive controls (double-distilled H_2O and DNA from cultures of *B. henselae*) were used in each PCR run. Products of the correct size were purified (QIAquick PCR Purification kit, Qiagen GmbH) and sequenced with the same primers, BhCS 781.p and BhCS1137.n., in both directions, with the Cy5/Cy5.5 Dye Primer Cycle Sequencing kit on a Long-Read Tower sequencer (Visible Genetics Inc., Toronto, Canada). Three hundred thirty-eight base-pair sequences of the *glt*A gene were obtained and compared with sequences of other known *Bartonella* species in GenBank by using the nucleotide BLAST program (National Center for Biotechnology Information; Available from: www.ncbi.nlm.nih.gov/BLAST/). Isolates identified as *Bartonella* species were obtained from 21 of the 70 blood cultures. All were isolated from *A. flavicollis,* and one was isolated from *Dryomys nitedula*. In addition, all were isolated from the first site (Nevrokopi village), and one was isolated from the second site (Pramanta village).

Within these 21 *Bartonella* isolates, eight genotypes were found. Among these isolates, one (AY435102 isolated from *A. flavicollis* trapped in Pramanta), was identical to ma106up strain, isolated from *Microtus agresti* (AF391789); another (AY435103 isolated from *A. flavicollis* trapped in Nevrokopi), was identical to af82up strain (AF391788), also isolated from *A. flavicollis* (3). Both strains ma106up (AF391789) and af82up (AF391788) were isolated in central Sweden (3) The rest of *Bartonella* isolates were from mammals trapped in Nevrokopi village and were divided into three phylogenetic groups. The first group, containing 10 isolates (AY435104–AY435113, isolated from *A. flavicollis*) and representing four novel genotypes, was 98% similar to *B. taylorii* (AF191502, isolated from *A. sylvaticus*) gltA gene. The second group, consisting of seven isolates that shared the same genotype (AY435114-AY435120 isolated from *A. flavicollis*), was 99% similar to *B. birtlesii* (AF204272 isolated from *Apodemus spp.*). The third group consisted of two isolates that shared the same genotype (AY435121 isolated from *D. nitedula*, and AY435122 isolated from *A. flavicollis*); this group was 97% similar to *B. grahamii* strain V2 (Z70016 isolated from *Neomys fodiens*).

This is the first study to identify *Bartonella* in small mammals in Greece. We found that 31.3% of the examined mammals were infected with *Bartonella* spp. The prevalence of culture-positive infections differed between the two sites (20/57 versus 1/13), although both are mountain areas with similar environmental and climatic conditions. A high prevalence of *Bartonella* infection in small mammals also has been described in other countries such as the United States (7) and Sweden (3), where 42.2% and 16.5% of the collected rodents were infected with *Bartonella* spp., respectively. As indicated in these studies, numerous *Bartonella* species are found in rodents. *A. flavicollis* was the most commonly captured species in Sweden (110/236), as well as in Greece (61/70). Identical *Bartonella* strains were isolated from *A. flavicollis* and *Microtus agresti* in both countries. Unlike Sweden, where the most frequent genotype was *B. grahamii,* in this study no isolate was identical to any *Bartonella* species known to cause human diseases. However, *B. elizabethae* was first isolated from a patient with endocarditis, and nothing was known concerning the organism's natural history until it was isolated from a rodent captured in Peru (4).

The occurrence and distribution of *Bartonella* in European hosts are

largely unknown. Given the existence of *Bartonella* spp. in every mammal group examined to date, the diversity of the genus is probably much greater than has been observed among the strains examined to date. In Greece, serologic evidence of human infection with *B. henselae* and *B. quintana* (8), has been found and a case of *B. quintana* endocarditis has been established (unpub. data). The public health relevance of *Bartonella* infections in small mammals in Greece compared with other countries remains to be defined.

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Reemerging Murine Typhus, Japan

To the Editor: Murine typhus is an arthropod-borne infectious disease caused by *Rickettsia typhi*, which is distributed widely around the world (1–4). In Japan, tsutsugamushi disease occurs most frequently in persons infected with rickettsioses (5). Spotted fever caused by *R. japonica* also occurs in the southwestern part of Japan (6,7). In the 1940s and 1950s, many murine typhus cases were reported in Japan. These diagnoses were made according to the clinical features of the illness and the reactivity of the serum samples to OX19 in Weil-Felix tests. A few cases were diagnosed on the basis of symptoms exhibited by animals infected with isolated rickettsiae and complement fixation tests, in addition to results of the Weil-Felix tests. The Weil-Felix test is useful for preliminary screening of rickettsiosis; however, the reaction could indicate epidemic typhus or spotted fever in some cases. Since 1958, only three murine typhus cases have been reported in Japan (8). In these cases, no serologic tests for epidemic typhus were conducted. Serum sample from patients with epidemic typhus and murine typhus frequently possess serologic cross-reactivity to *R. typhi* and *R. prowazekii*, respectively (9). Thus, the possibility of epidemic typhus could not be excluded definitively in these cases.

On May 4, 2003, a 56-year-old man living in Tokushima, Japan, sought medical care; he had a temperature of 39.1°C and exanthema on the trunk and the upper limbs. No surface lymph nodes were palpable. He was treated with lincomycin and cefditoren pivoxil with no improvement. On day 3, the patient informed caregivers that he had been in a bamboo grove on days 1 and 11 before the onset of symptoms. C-reactive protein of the serum sample collected on day 3 was positive (= 7.6 mg/dL). From this finding, spotted fever was suspected; the disease is endemic in Tokushima. On day 4, the exanthema had spread systemically, and treatment with minocycline was started, which led to a gradual decrease in fever and rashes. The patient was admitted to the Tokushima University Hospital on day 6 of the illness for diagnosis and further treatment.

Serum samples were collected from the patient on days 5, 6, 9, 20, and 34. Indirect immunoperoxidase tests on the serum samples for tsutsugamushi disease, spotted fever, murine typhus, and Q fever on day 5 of the illness were negative for immunoglobulin (Ig) G and IgM antibodies (<1:40). Weil-Felix tests on the serum samples on days 5 and 9 of the illness were negative for OX2, OX19, and OXK. Indirect immunofluorescence of the serum samples on days 6, 9, 20, and 34 of the illness was conducted by using strains 18 and Wilmington of *R. typhi*, and the strain Breinl of *R. prowazekii* as typhus group rickettsiae; and the strain YH of *R. japonica*, the strain Malish 7 of *R. conorii*, and the strain Tick of *R. montanensis* as the spotted fever group rickettsiae. All serum samples tested for the rickettsiae showed an IgM titer of 1:20. On the other hand, the IgM titers of these serum samples, to the *Orientia tsutsugamushi* were <1:20.

For the IgG antibodies of these serum, spotted fever group rickettsiae were negative (<1:20). However, the typhus group rickettsiae were positive for IgG antibodies. Among the typhus group rickettsiae, strain 18 of *R. typhi* had the highest elevated titers. The titers to the sera on days 6, 9, 20, and 34 of illness were 1:80, 1:160, 1:160, and 1:80, respectively. Another strain of *R. typhi*, the strain Wilmington, had lower titers of 1:40, 1:80, 1:80, and 1:40, on days 6, 9, 20, and 34 of the illness, respectively. This could have occurred because strain 18 may be a closer antigenic relation of the causative agent than is the strain Wilmington. *R. prowazekii* demonstrated the lowest IgG titers among typhus group rickettsiae for these serum samples, <1:20, 1:20, 1:40, and 1:20, on days 6, 9, 20, and 34 of the illness, respectively. These results suggested that the disease was murine typhus.

To demonstrate more detailed antigenic reactivity, Western immunoblotting of rickettsiae was conducted by using a serum specimen from day 20. All of the rickettsiae were reactive to the serum to various extents. The serum reacted to the ladder-like lipopolysaccharide of *R. typhi* and *R. prowazekii*; the antigenicity of rickettsial lipopolysaccharide is group-specific. As expected from the immunofluorescence data, no reaction was demonstrated to the lipopolysaccharide of spotted fever group rickettsiae, *R. japonica* and *R. montanensis*, although trace cross-reactivity, mainly to rOmpB, was shown. Thus, typhus group rickettsiosis was suspected for this case on the basis of these data. Compared to the trace reaction of spotted fever group rickettsiae to rOmpB, a stronger, but still weak, reaction was detected to the heat-labile state of rOmpB of *R. prowazekii,* and an extremely strong reaction was demonstrated to the heat-labile and heat-stable states of rOmpB of *R. typhi*. These results strongly suggested that the disease was murine typhus.

To confirm this diagnosis, we conducted absorption tests as described previously (10). The patient serum collected on day 20 showed complete absorption by the homologous antigen, the purified *R. typhi* strain 18, demonstrating no reaction to *R. typhi* or to *R. prowazekii* by immunofluorescence. However, the serum showed incomplete absorption by the heterologous antigen, the purified *R. prowazekii*, demonstrating no reactivity to *R. prowazekii* but some reactivity to *R. typhi*. These tests confirmed the diagnosis of murine typhus.

Murine typhus has never been reported in Japan after the 1950s, except for the three suspected cases and this case. Although other undiagnosed cases may have occurred, they appear to be few; many febrile cases of exanthema have been examined for various rickettsioses, especially after spotted fever was diagnosed in Japan in 1984. Murine typhus may have reemerged because of the recent increase of black rats, *Rattus rattus,* in Japan. This patient mentioned that he had captured a rat and disposed of the carcass about a week before the onset of symptoms. Infection could have resulted at that time from contamination with feces of infected fleas such as the oriental rat flea, *Xenopsylla cheopis*. Historical review indicates that this is the first complete serologic diagnosis of a murine typhus case in Japan.

Acknowledgment

We thank Akio Adachi for his valuable suggestions.

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Cutaneous Leishmaniasis, Northern Afghanistan

To the Editor: In Afghanistan, most cutaneous leishmaniasis cases are caused by *Leishmania tropica*, which is transmitted anthroponotically by the sandfly *Phlebotomus sergenti* (1). Cutaneous leishmaniasis can have devastating effects on local communities because of its clinical symptoms, i.e., large, multiple, or both, disfiguring lesions, that can lead to social ostracism of affected persons (e.g., women are often deemed unsuitable for marriage or to raise children) (2). Cutaneous leishmaniasis is considered a low priority disease by international donor agencies because treatment costs are high and the disease does not cause death (3).

Data on the effects of cutaneous leishmaniasis in Afghanistan previously have been available only for Kabul city; recent studies have reported an estimated 67,500 cases (4). Because of the migration of an estimated 4.5 million infected Afghan refugees returning home from other countries, the sporadic treatment of patients infected with cutaneous leishmaniasis, and limited control of the sandfly vector, *L. tropica* has spread to areas that were previously nonendemic for the disease, e.g., northeastern Afghanistan.

A survey in Faizabad city, Badakhshan Province, was conducted in June 2003 by HealthNet International to collect data on the impact of cutaneous leishmaniasis. Leishmaniasis in this region is transmitted from April to October. The city was divided into 10 districts, and 20 households were surveyed along a randomly chosen transect drawn from the center of each district. A team of experienced medical staff clinically diagnosed cutaneous leishmaniasis (based on the presence or absence of cutaneous leishmaniasis lesions or scars, number of lesions, date of lesion onset) in household members and interviewed them to collect demographic data (gender, age). Because of logistic constraints, parasitologic diagnosis of cutaneous leishmaniasis lesions (i.e., microscopic examination or parasite culture) was not conducted. However, in Afghanistan, skin lesions attributed to causes other than cutaneous leishmaniasis are rare, and experience has shown 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 Ministry of Health. Informed consent was obtained from study participants; all study participants with active cases of the disease were offered free anti-leishmanial treatment at the HealthNet International leishmaniasis clinic.

We surveyed 1,832 people from 200 households; 8.3% (152/1,832) and 7.8% (142/1,832) had active cutaneous leishmaniasis lesions or scars, respectively. Of those persons with cutaneous leishmaniasis lesions, the mean lesion number was 2.4 (range 1–14), the mean lesion size was 2.4 cm (range 1–5.5), and the mean lesion duration (to survey date) was 5.6 months (range 1–11). Active prevalence was not associated with gender (Yates-corrected $\chi^2 = 2.16$, $p = 0.14$); 85/152 (56%) of the cutaneous leishmaniasis case-patients were women, and 67/152 (44%) of cutaneous leishmaniasis case-patients were men. Data showed that persons aged ≤15 years were at higher risk of contracting the disease than were persons aged >15 years (odds ratio = 2.23, 95% CI 1.54 to 3.24, Yates-corrected $\chi^2 = 19.44$, $p < 0.001$).

Based on population estimates of 65,000 people and observed prevalence, approximately 5,395 cutaneous leishmaniasis case-patients would be found in Faizabad. The low prevalence of scars, compared to the high prevalence of disease, shows that cutaneous leishmaniasis has been introduced into Faizabad only recently (1,4). Local Ministry of Health records show that the disease was virtually absent (<50 annual cases) in Badakhshan 3 years ago; this information is corroborated by the observation that the mean time since the recovery of surveyed people with cutaneous leishmaniasis scars was 1.5 years (range 0.3–15). Although no attempts were made to identify circulating *Leishmania* sp., the current epidemic is likely caused by *L. tropica* because both men and women are equally affected and younger age groups are at higher risk for cutaneous leishmaniasis than older age groups (1,4). Current analyses are under way to establish risk factors (e.g., presence or absence of animals, type of house construction) for contracting the disease.

With support from HealthNet International, three leishmaniasis clinics have been established in Faizabad to increase the total number of patients whose illness is diagnosed and treated; to reduce the risk to susceptible persons through the subsidized sale of insecticide-impregnated bed nets; to train and supervise the Ministry of Health staff in diagnosis, treatment, and prevention of the disease; and to implement health education campaigns for patients attending the clinics and the community at large. Hopefully, these activities will prevent the current cutaneous leishmaniasis outbreak from becoming an epidemic, as it has been in Kabul over the past 15 years (4,5).

Acknowledgments

We thank the Afghan Ministry of Health and the HealthNet International survey team staff for logistical support.

This study was supported by the United Nations Mission to Afghanistan.

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Rickettsia felis, *Bartonella henselae*, and *B. clarridgeiae*, New Zealand

To the Editor: The cat flea (*Ctenocephalides felis felis* Bouché, 1935) is a ubiquitous parasite of domestic and wild animals that also feeds readily on people. Recent studies have implicated the cat flea as a vector of new and emerging infectious diseases (1). To determine the pathogens in *C. felis* in New Zealand, we collected 3 cat fleas from each of 11 dogs and 21 cats at the Massey University Veterinary Teaching Hospital from May to June 2003. The fleas were stored in 95% alcohol until they were identified by using morphologic criteria and washed in sterile phosphate-buffered saline. The DNA from each flea was extracted individually by using the QiaAmp Tissue Kit (QIAGEN Ltd., Hilden, Germany), according to the manufacturer's instructions. When polymerase chain reaction (PCR) was performed with primers for *glt*A and *rOmp*A as described (2), products were obtained with DNA from 15 (15%) of the fleas. The sequences of the products were identical to those of *Rickettsia felis* (GenBank AF191026) with infected fleas taken from both dogs (3/11; 27%) and cats (7/21; 33%). When PCR was performed with primers for the 16S-23S rDNA interspacer region as described (3), products were obtained with DNA of four fleas. The sequences of the products from three fleas (from two cats) were identical to that of *Bartonella henselae* (GenBank AF312495), and the sequence of the product of one flea (from a cat) was identical to that of *B. clarridgeiae* (GenBank AF167989).

Our study is the first to identify *R. felis* in Oceania. The organism is a recently described human pathogen, and infections with this spotted fever group rickettsia have already been reported in 11 persons: 4 persons in the United States, 2 persons in Brazil, 4 persons in Europe, and 1 person in Thailand. The symptoms of the patients were nonspecific and included fever, headache, and rash. Diagnoses were made by sequencing products obtained by PCR with primers for the 17-kDa protein (4), citrate synthase (4), and PS 120 protein (5) genes. *R. felis* has been established in tissue culture (XTC-2 and Vero cells) (6), and serologic testing has been used to diagnose infections (5). Reports indicate that patients respond rapidly to doxycycline therapy (5), and in vitro studies have shown the organism is susceptible to rifampin, thiamphenicol, and fluoroquinolones.

B. henselae is an agent of cat-scratch disease, bacillary angiomatosis, bacillary peliosis, endocarditis, bacteremia, and various neurologic and ocular conditions. Cats are the reservoir hosts, and contact with cats and their fleas is an established risk factor for most infections. Although *B. henselae* has been isolated from 17% of domestic cats in New Zealand (7), only two human infections have been reported in the country; neuroretinitis was diagnosed in both patients (8). In neighboring Australia, however, cat-scratch disease, bacillary angiomatosis, and endocarditis have been diagnosed in numerous patients. Cats are also the reservoir hosts of *B. clarridgeiae* which has been implicated as an agent of cat-scratch disease in humans and aortic valve endocarditis and hepatic disease in dogs (9). The organism has been found in cat fleas (as great as 17%) in Europe (1), and although we found only one flea infected with *B. clarridgeiae* in New Zealand, this description is the first of the organism in Oceania. However, *B. clarridgeiae* has been found in domestic cats in nearby Indonesia and the Philippines (10).

Our findings add to the accumulating data on *R. felis*, *B. henselae,* and *B. clarridgeiae* and should alert medical workers in New Zealand, a common tourist destination, to the possibility that their patients may be infected with these organisms.

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Enterohemorrhagic *Escherichia coli* O157, Kinshasa

To the Editor: During the rainy season, from April to September 2003, 463 children ≤15 years of age (median 10 months) with severe diarrhea were admitted to the Pediatric Hospital of Kalembelembe in Kinshasa, the capital of the Democratic Republic of Congo. The population of the outbreak area was approximately one million.

Several children with bloody diarrhea without fever were treated. They came from six districts of Kinshasa (Bumbu, Selembao, Makala, Kimbanseke, Masina, and Ndjili). Abdominal cramps, nausea, vomiting, and dehydration were uncommon. The duration of illness ranged from 5 days to 2 weeks. Available antiparasitic drugs, trimethoprim-sulfamethoxazole, and ampicillin showed no effect against the illness. Fifty-six infants died between June and July. Symptoms of hemolytic uremic syndrome developed in most of them.

Stool samples from 32 patients were screened for parasites, enteropathogenic bacteria, rotavirus, and adenovirus. Three samples were positive for rotavirus. In contrast, all stool cultures were positive for *Escherichia coli* which always grew as pure cultures on purple bromocresol agar, a nonselective medium containing lactose. The *E. coli* isolates appeared sorbitol negative when tested on MacConkey sorbitol; they were agglutinated by O157 and H7 antisera (Difco Laboratories, Detroit, MI) and lacked expression of β-glucuronidase. All *E. coli* isolates were sent to the Pasteur Institute in Bangui, Central African Republic, for further characterization. Polymerase chain reaction allowed detection of Shiga-like toxin *slt-1* and *slt-2* genes (1,2) in isolates from all patients. The Vero cell assay phenotypically confirmed cytotoxicity of these isolates, with most of them being seroneutralized by rabbit antisera against Shiga toxin (3). Thus, all *E. coli* isolates responded to the definition of enterohemorrhagic *E. coli*.

Before 2003, sporadic infections or outbreaks caused by enterohemorrhagic *E. coli* were not reported as a cause of bloody diarrhea in the Democratic Republic of Congo. A case-control study could not be performed because of political unrest in Kinshasa. Although reported outbreaks of *E. coli* O157 in sub-Saharan Africa have been few to date, available information indicates that the pathogen has wide geographic distribution. *E. coli* O157–related diarrhea outbreaks that occurred before 2003 have been reported in South Africa, Swaziland (4), and Malawi (5) in 1992; Central African Republic (6) and Kenya (7) in 1996; Cameroon in 1998 (8); and Nigeria (9) and Ivory Coast (10) in 2000. In the Central African Republic and in Zémio, a small village located on the Democratic Republic of Congo border, outbreaks of bloody diarrhea in 1996 were attributed to *E. coli* O157 from molecular test results (6).

Since 2001, an increasing number of cases of acute bloody diarrhea have been reported in Kinshasa between June and August. During this 2003 outbreak, an investigation could not be conducted; possible routes of transmission would include person-to-person contact related to lack of hygiene, and contaminated food and water.

In 1996 in the Central African Republic and in 1998 in Cameroon, the major contributing factors of the *E. coli* O157 outbreak were consumption of smoked zebu meat and contaminated drinking water. Studies of *E. coli* O157 carriage rates among livestock, food, and environment in this central African area might be useful in assessing the potential for future outbreaks.

Hemolytic uremic syndrome occurs in approximately 8% of children and an unknown proportion of adults infected with *E. coli* O157 and can be fatal without hemodialysis. The high death rate of infants during this outbreak was linked to the lack of treatment (mainly hemodialysis) at the beginning of the epidemic. Obviously, more work is needed to better define the incidence and epidemiology of *E. coli*–associated diarrhea in the Democratic Republic of Congo so that optimal recommendations for preventing and managing illness can be developed.

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Iatrogenic *Mycobacterium simiae* Skin Infection in an Immunocompetent Patient

To the Editor: We report a case of a 36-year-old woman who sought treatment for 45 firm and erythematous nodular lesions on her face and neck. A physical examination showed no other abnormalities. Results of a chest x-ray and routine laboratory tests were normal. The patient tested negative for hepatitis B and HIV. Three weeks before she sought treatment, the patient reported receiving multiple intradermal microinjections in her face and neck for cosmetic purposes (mesotherapy) with an unlicensed product consisting of a solution of glycosaminoglycans. The injections had been administered by an unlicensed practitioner in a nonmedical office setting. The patient stated that 2 days after the therapy, a fever developed; it persisted for several days, along with redness at the inoculation sites, which gradually developed into nodules.

Standard staining of a biopsied specimen from the lesion site was negative for bacteria, fungi, and mycobacteria. A histopathologic examination of a biopsy specimen showed an unspecific granulomatous infiltrate. Culture for common bacteria and fungi was negative, but culture of a sterile nodule aspirate on Lowenstein-Jensen medium was positive for acid-fast bacteria after 5 weeks. By using restriction endonuclease analysis of the 65-kDa heat shock protein gene (1), we found that the isolate showed a pattern compatible with *Mycobacterium simiae*. Identification was subsequently confirmed by high performance liquid chromatography of mycolic acids at the Centers for Disease Control and Prevention, Atlanta, Georgia. The isolate was tested for drug susceptibility against a panel of drugs and found to be resistant to most drugs tested (streptomycin, isoniazid, rifampin, ethambutol, ethionamide, rifabutin, ciprofloxacin, kanamycin, capreomycin, p-aminosalicylic acid, ofloxacin, and amikacin) and susceptible to clarithromycin at an MIC of 1 μg/mL. Treatment with clarithromycin was started, and the granulomas slowly cleared after 9 months of treatment.

To our knowledge, this is the first reported case of an iatrogenic skin infection caused by *M. simiae* in an immunocompetent person. *M. simiae* is a species of nontuberculous mycobacterium commonly found in nature, but its role as a pathogen has been controversial. The slow-growing, photochromogenic mycobacterium has been isolated from both surface and tap water and has been associated with a nosocomial pseudo-outbreak suspected to have originated from a contaminated hospital water

supply (2). *M. simiae* rarely causes disease in immunocompetent patients; most infections are associated with AIDS patients. (3–5).

Although this patient responded to treatment with clarithromycin, no established optimal therapeutic regimen exists against this species of *Mycobacterium*. *M. simiae* is often multidrug resistant, but successful therapy with clarithromycin in combination with ethambutol and ciprofloxacin has been reported in AIDS patients (6,7).

We conclude that *M. simiae* can cause skin infections if injected directly into the dermis. Prolonged treatment is necessary to cure the patient of the infection. This report underscores the risk from alternative therapies performed with unlicensed products and by unlicensed practitioners. Unusual infectious agents should be considered when diagnosing skin infection in patients who have received injections for cosmetic purposes.

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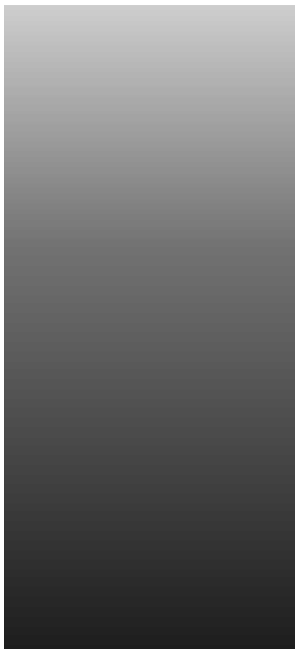

Brief summaries of articles on pertinent emerging issues published elsewhere.
Edited by Joseph E. McDade

POPULATION GENETICS

Population Genetics of *Cryptosporidium parvum*

Research on the population genetics of *Cryptosporidium parvum* is showing exciting developments. A study of numerous *C. parvum* isolates of human and animal origin collected in a small area of Scotland has uncovered differences in the epidemiology of what was originally referred to as *C. parvum* type 1 (also, anthroponotic type) and type 2 (zoonotic type), a designation recently changed to *C. hominis* and *C. parvum*. This study, based on fingerprints obtained from eight polymorphic genetic markers, showed striking differences between the population structure of these species; a clonal *C. hominis* population and a more complex *C. parvum* population. The population structure of *C. hominis* was consistent with the epidemic nature of human infections, where a small number of genotypes predominate. In contrast, *C. parvum* (type 2) genetic fingerprints showed evidence of random mating among genetically diverse parasites. Evidence for partitioning of the species according to host (human and bovine) was also inferred from these data. Two questions we are left with are whether these observations are specific to this particular location, where human transmission is relatively infrequent, and whether regions with high prevalence of human cryptosporidiosis will show more complex structures in both species. If substructuring into human– and bovine–derived *C. parvum* (type 2) is consistently observed, the potential for zoonotic transmission of *C. parvum* may also have to be reexamined.

Widmer G. Population genetics of *Cryptosporidium parvum*. Trends Parasitol 2004;20:3–6;discussion 6. Available at: http://dx.doi.org/10.1016/j.pt.2003.10.010

DIAGNOSTIC VIROLOGY

Role for Arrays in Clinical Virology: Fact or Fiction?

Polymerase chain reaction (PCR) detection of genomic DNA or RNA has become an indispensable tool for the diagnosis and surveillance of viral disease. Perhaps the biggest drawback of PCR, though, is that only one or a few viruses can be searched for in a single test. DNA chips or microarrays have the potential to overcome this disadvantage and can provide a near-patient test that identifies both known viruses and those causing newly emerging diseases such as SARS. For this potential to be realized, however, the PCR techniques capable of amplifying any adventitious sequence in a clinical specimen and the microarraying hybridization and detection technologies necessary for obtaining rapid and reproducible results need to converge. The arrays that have already been developed for use in virology point the way forward.

Clewley JP. A role for arrays in clinical virology: fact or fiction? J Clin Virol 2004; 29:2–12. Review. Available at: http://dx.doi.org/10.1016/j.jcv.2003.08.002

MOLECULAR EPIDEMIOLOGY

Multilocus Sequence Typing and the Evolution of Methicillin-resistant *Staphylococcus aureus*

Methicillin-resistant *Staphylococcus aureus* (MRSA) continue to adapt to the selective pressure of antimicrobial agents and to exploit new niches, as evidenced by the recent isolation of strains with high-level vancomycin resistance and the emergence of MRSA as a community pathogen. The combined use of the bacterial genotyping technique, multilocus sequence typing (MLST), and characterization of the mobile methicillin-resistance determinant, staphylococcal chromosomal cassette *mec* (SCC*mec*), has provided new insights into MRSA strain nomenclature, evolution, and epidemiology. The first MRSA emerged when SCC*mec* was acquired by an epidemic methicillin-susceptible strain prevalent in Europe. Acquisition of SCC*mec* by other successful strains has led to the emergence of at least 11 major epidemic MRSA strains belonging to five distinct lineages with a global geographic distribution. These five lineages have evolved both hospital-acquired and community-acquired MRSA, but some of the newly emerging community strains descend from other lineages.

Robinson DA, Enright MC. Multilocus sequence typing and the evolution of methicillin-resistant *Staphylococcus aureus*. Clin Microbiol Infect 2004;10:92–7.

VACCINES

Mimicking Live Flavivirus Immunization with a Noninfectious RNA Vaccine

A new genetic vaccine against flaviviruses is presented that can stimulate a comprehensive immune response similar to a live vaccine but with the safety profile of an inactivated vaccine. The principle is based on the use of noninfectious but replication-competent genomic RNA. This vaccine mimics live viral infection, although there is no

spread of virus in the body. The complete replication complex is expressed, including all viral nonstructural proteins known to be targets of the humoral and cellular immune response. In addition, subviral particles consisting of the viral surface proteins prM/M and E are produced and presented to the immune system. The proof-of-principle is demonstrated with tick-borne encephalitis virus (TBEV). Because of the close genetic relationship among members of the genus *Flavivirus*, this principle presumably can also be applied to other pathogens of worldwide medical importance, such as dengue viruses, Japanese encephalitis virus, yellow fever virus, and West Nile virus. The vaccine consists of in vitro synthesized genomic RNA, genetically modified to abolish viral infectivity but to provide ample production and release of subviral particles. Gene-gun mediated injection of this experimental TBEV vaccine into adult mice is shown to yield a neutralizing and protective immune response.

Koffler RM, Aberle JH, Aberle SW, Allison SL, Heinz FX, Mandl CW. Mimicking live flavivirus immunization with a non-infectious RNA vaccine. Proc Natl Acad Sci U S A 2004;101:1951–6. Epub 2004 Feb 09. Available at: www.pnas.org/cgi/content/abstract/101/7/1951

ECOLOGY

Bacterial Biofilms: Prokaryotic Adventures in Multicellularity

Three-dimensional bacterial biofilm microstructures display multicellular characteristics in common with higher organisms, such as cell death and differentiation during development, which carry several medical and evolutionary implications. Biofilm microstructures appear to enhance bacterial tolerance to a number of stresses, including antimicrobial agents; determinants of multicellularity, such as cell-cell signaling, are necessary for this tolerance. Processes of microcolony development and differentiation are therefore of particular interest as targets for novel strategies to control biofilms.

Webb JS, Givskov M, Kjelleberg S. Bacterial biofilms: prokaryotic adventures in multicellularity. Curr Opin Microbiol 2003;6:578–85. Review. Available at: http://dx.doi.org/10.1016/j.mib.2003.10.014

PATHOGENESIS

CCR5 and CXCR4 Co-Receptors in HIV Infection

This article examined the central role played by co-receptor expression and usage in the transmission and pathogenic effects of HIV-1 infection of humans. The review contains a discussion of the HIV-1 phenotypic variants defined by their use of the CCR5 or CXCR4 co-receptors. How the different cellular tropism patterns of these viral variants influence how and where HIV-1 replicates in vivo is also discussed, with emphasis on the thymus and gut-associated lymphoid tissues. The review also contains a consideration of the possible outcomes of the use of co-receptor antagonists as drugs to treat HIV-1 infection in vivo.

Moore JP, Kitchen SG, Pugach P, Zack, JA. The CCR5 and CXCR4 coreceptors—central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. AIDS Res Hum Retroviruses 2004;20:111–26.

Six Modern Plagues and How We Are Causing Them

By Mark Jerome Walters

Published by Shearwater Books, Washington, DC, USA, 2003

ISBN: 155963992X
Pages: 206, Price: US $22.00

It's not the price but rather the pathology of progress that author Mark Walters laments in Six Modern Plagues. Weaving anecdote with theory, Walters draws from his diverse backgrounds in veterinary medicine and journalism to link ecologic tampering to some of the most featured—if not feared—diseases of our time.

In recounting the origin of bovine spongiform encephalopathy (BSE) or "mad cow disease," the author describes how, in compounding cattle feed with slaughterhouse byproducts, we converted our oldest domesticated herbivores into meat eaters. Bovine trickery aside, Walters renders his frank assessment that, "violating such evolutional boundaries can seem unnatural if not disgusting." He goes on to add a damaging link to the food chain, leading to >100 human cases of always-fatal variant Creutzfeldt-Jackob disease (vCJD). Most cases occurred in or are related to the United Kingdom, where, by late 2000, more than 35,000 herd of cattle were infected with BSE. Though the practice of supplementing feed with animal byproducts has, for the most part, been abandoned, Walters suggests that certain risks remain, as prions, the subviral infectious agent responsible for mad cow and vCJD are also found in wild game, though, to date, no one has connected consumption of deer or elk meat with vCJD.

However, far from being out of the woods, humankind remains vulnerable to exotic diseases from unlikely sources. Walters attributes the rise of Lyme disease to fragmented forests. Dissected by roads and separated by developments, eastern woodlands can no longer sustain large natural predators, but they remain ideal habitats for deer and mice, which can expose humans to ticks carrying the dangerous Lyme spirochete. An ocean away, as forays into sub-Saharan Africa tempted settlers to add bush meat to their sparse diets, HIV made the species jump, Walters suggests, propelling a worldwide AIDS pandemic.

Beyond our abuse of nature, Walters cites antimicrobial misuse as a precipitator of frightening disease. He focuses most on antimicrobial agents in animal feed, accusing policymakers of ignoring the threat of antimicrobial resistance, fearing more the resistance of agricultural interests bent on nurturing their flocks with medicated rations.

The foundation for the author's discussion varies from rock-solid to rickety, but his half-dozen arguments portray a society more absorbed in immediate gratification than in ultimate consequence. With that, he offers a guarded prognosis that depends on both our cleverness at finding new cures and our commitment to restoring ecologic wholeness.

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Francisco José de Goya y Lucientes (1746–1828). Self-portrait with Doctor Arrieta (1820)
Oil on Canvas (114.62 x 99.38 x 9.53 cm). The Minneapolis Institute of Arts, The Ethel Morrison Van Derlip Fund

"Goya in gratitude to his friend Arrieta for the skill and care with which he saved his life in his acute and dangerous illness suffered at the end of the year 1819 at the age of 73. He painted it in 1820" (1) reads the inscription at the bottom of Goya's self-portrait on this month's cover of Emerging Infectious Diseases. An affirmation of medical practice, the painting is also an acknowledgment of human compassion, a quality the artist thought extremely rare.

Conflicted in his acceptance of the world and in his portrayal of it and deeply mistrustful of human nature, Goya lingered on the dark side as he painted the full spectrum of life experiences (2,3). During his long artistic career, he dwelled on the tensions of Spanish society of his day, whose institutions, including medicine, he gleefully satirized (e.g., Of What Illness Will He Die?) (4).

Deaths in his family and debilitating illness throughout his years often interfered with his work and left him weak and disillusioned. "Neither sight, nor pen nor inkwell; all these I lack and all that is plentiful is my will," the painter remarked to a friend regarding his loss of hearing, poor health, and frail disposition (2). Near the end of his life, once again he became seriously ill. Overcoming his natural aversion to authority, he entrusted himself to the care of a physician friend. When the health crisis subsided, Goya created Self-portrait with Doctor Arrieta.

Unlike most paintings of his later years, which evoke horror and darkness, this double portrait imprints a gentle aspect of humanity on the mild physiognomies of physician and patient. Even so, rather than a departure from his sinister worldview, the painting of one man tending to another was a gesture of gratitude after deliverance from death (5).

The portrait is an empathetic rendition not of Goya alone but of the universal human patient. Isolated but for the intruding shadows witnessing his pending demise, in a drab dressing gown, generic, exposed, and vulnerable, Goya embodies the plight of the sick. Withered and limp, unkempt and undignified, he is reduced to an infantile state, to be comforted and cajoled, humored with therapeutic potions and measures, and ordered to obey.

Gone is the thundering presence, the compelling personality, the artistic genius, the signature mistrust of human nature. Opinions and attitudes were shed at the sickroom door, along with his everyday clothes and his ability to walk and control his life. With his private condition on public display, he is at the mercy of his caretakers. Clutching the carmine blanket between him and the world, he succumbs to the physician's sympathetic embrace and, near death, sinks deeper into isolation.

The kindly physician is warm and obliging if not unduly hopeful. Aware of his limited capacity to reverse the course of illness, he focuses on what is within his capacity, comfort and support. He draws near the patient, as if to become one with him and propel his own strength and energy onto the ailing body. The closeness of his embrace equals his instinct to alleviate pain and his oblivion of risk to himself from proximity to the patient. As he firmly administers the medication, his face wears the look of the stoic philosopher and the eagerness of the medical intern.

An astute observer of the human condition, Goya understood the tragic nature of disease, often manifested in our inability to prevent its onset, control its course, and predict its outcome. Understanding of infection has burgeoned since 1820, yet patient isolation, vulnerability, uncertainty, and untimely death remain unresolved. In emerging disease puzzles, where treatment is sometimes administered while large pieces are still being assembled, the old measures of infection control and quarantine are challenged by new environmental, social, and scientific developments. Contagion, unknown to Dr. Arrieta, is particularly pertinent in diseases like SARS (6), where the threat is not fully quantified until, unlike the images in Goya's double portrait, the patient and the caretaker are one.

Polyxeni Potter

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Upcoming Infectious Disease Activities

May 1–4, 2004
14th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID)
Prague, Czech Republic
Contact: Administrative Secretariat
+44 61 686 77 11
email: info@akm.ch
Web site:
http://www.akm.ch/eccmid2004/

May 11–14, 2004
38th National Immunization Conference
Nashville, TN
Contact: Suzanne Johnson-DeLeon
404-639-8225
email: NIPNIC@cdc.gov

May 31–June 3, 2004
II International Congress of Dengue and Yellow Fever
Convention Center
Havana, Cuba
email:
congreso.dengue@infomed.sld.cu
Web site: http://www.cidfa2004.sld.cu

June 3–5, 2004
13th International Symposium on HIV & Emerging Infectious Diseases
Toulon, France
Web site:
http://www.avps.org/2003/hiv.htm

June 28–30, 2004
2004 Annual Conference on Antimicrobial Resistance
Bethesda, MD
Contact: 301-656-0003
email: resistance@nfid.org
Web site: http://www.nfid.org/conferences/resistance04

September 19–23, 2004
Extremophiles 2004
American Society for Microbiology
Cambridge, MD
Web site:
http://www.asm.org/Meetings/index.asp?bid=19177

EMERGING INFECTIOUS DISEASES
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol. 10, No. 6, June, 2004

Upcoming Issue

Look in the June issue for the following topics:

Chronic Wasting Disease of Deer and Elk

Rotavirus Surveillance in Asia

Salmonella-based Rodenticides

Airborne Infection with *Bacillus anthracis*

Indirect Prion Transmission in Mule Deer

Bacillus anthracis Recovery from Nonporous Surfaces

SARS Origin in China

Mycobacterium ulcerans, Australia

Bovine Spongiform Encephalopathy in Greater Kudu

Sporadic Cryptosporidiosis, England, 1996–2000

Environmental Exposure and Leptospirosis, Peru

Nursing Home Residents and *Enterobacteriaceae*

Campylobacter Infections in Two Danish Counties

Complete list of articles in the June issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Editorial Policy and Call for Articles

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

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

Emerging Infectious Diseases is published in English Chinese, French, and Spanish translations of some articles can be accessed at http://www.cdc.gov/eid/ncidod/EID/trans.htm. The journal features the following types of articles: Perspectives, Synopses, Research Studies, Policy and Historical Reviews, Dispatches, Commentaries, Another Dimension, Letters, Book Reviews, and News and Notes. The requirements of each type of article are described in detail below and at http://www.cdc.gov/eid/ncidod/EID/instruct.htm. To expedite publication, we post journal articles on the Internet as soon as they are edited.

Instructions to Authors

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

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

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

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

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

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

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

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases website (www.cdc.gov/eid).

Types of Articles

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

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

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, 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), a one-sentence summary, and 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 should contain no more than one figure or table. References (no 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.