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Emerging Infections in Africa

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September 2006

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Daudi E.S. Tingatinga.
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(73.66 cm × 73.66 cm)
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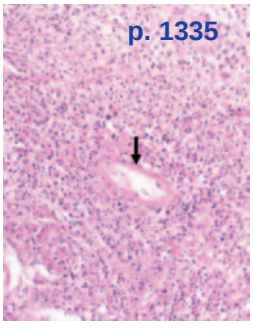

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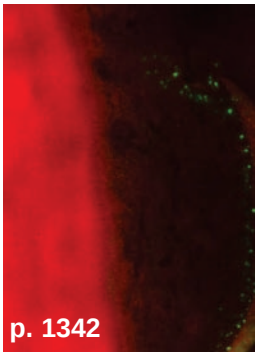

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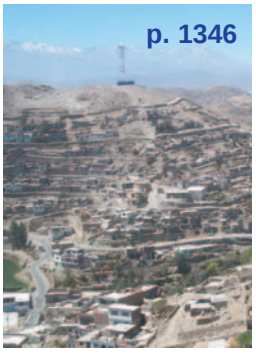
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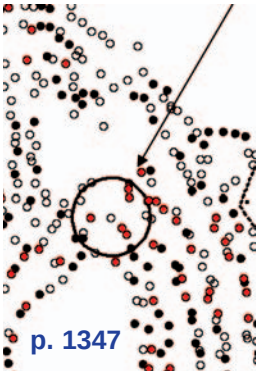
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Nosocomial Tuberculosis in India

Madhukar Pai,*† Shriprakash Kalantri,† Ashutosh Nath Aggarwal,‡ Dick Menzies,§ and Henry M. Blumberg¶

Most high-income countries implement tuberculosis (TB) infection control programs to reduce the risk for nosocomial transmission. However, such control programs are not routinely implemented in India, the country that accounts for the largest number of TB cases in the world. Despite the high prevalence of TB in India and the expected high probability of nosocomial transmission, little is known about nosocomial and occupational TB there. The few available studies suggest that nosocomial TB may be a problem. We review the available data on this topic, describe factors that may facilitate nosocomial transmission in Indian healthcare settings, and consider the feasibility and applicability of various recommended infection control interventions in these settings. Finally, we outline the critical information needed to effectively address the problem of nosocomial transmission of TB in India.

The risk that *Mycobacterium tuberculosis* can be transmitted from patients with active tuberculosis (TB) to other patients and healthcare workers has been recognized for many years (*1*). The level of risk varies by setting, occupation, patient population, and effectiveness of TB infection control measures (*2–5*) but is higher in facilities that manage large numbers of smear-positive TB patients who do not receive rapid diagnosis, isolation, and treatment, particularly in the absence of other infection control measures (*2–5*). A hierarchy of control measures, including administrative, engineering, and environmental controls and personal protection measures, has been recommended to reduce nosocomial TB risk (*2*,*3*,*5*,*6*). These recommended measures are implemented by healthcare facilities in high-income countries (*3*,*6*), but given their high cost, few facilities in low-income countries can afford to implement them.

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The World Health Organization (WHO) has proposed practical and low-cost interventions to reduce nosocomial transmission in settings where resources are limited (*7*). These recommendations emphasize prompt diagnosis and rapid treatment of TB rather than expensive technologies, such as isolation rooms and respirators. However, despite the widespread implementation of the directly observed therapy, short course (DOTS) strategy, which is internationally recommended, compliance with these simpler guidelines is generally poor in low-income countries (*8*).

In general, the primary focus of national TB programs in high-prevalence, low-income countries is to expand basic DOTS services. Typically, nosocomial transmission is ignored, given countries' limited resources, but several factors illustrate that nosocomial TB must be addressed, even in such areas. First, nosocomial transmission is of concern because it affects not only patients who are exposed but also the healthcare workforce, which could adversely affect healthcare services over time (*7*). Second, transmission of TB can have serious consequences, particularly with multidrug-resistant TB (MDRTB). Several outbreaks in the United States demonstrated the role that hospitals can play as focal points of MDRTB transmission (*9–13*), a phenomenon also seen in Europe, South America, South Africa, and Russia (*14–16*). These outbreaks can be explosive and associated with high death rates because hospitalized patients are often immunocompromised (*2*,*9*). Therefore, interventions to reduce nosocomial transmission of TB are useful and cost-effective preventive measures to control TB, including MDRTB, particularly in tertiary care settings.

Third, nosocomial TB must be addressed because it can help the healthcare system, particularly the private health sector, improve TB diagnosis and treatment and better align practices with the DOTS strategy. For example, detecting smear-positive TB with microscopy is a key component of the DOTS strategy and an important administrative infection control measure. However, several studies have shown that private practitioners in India tend to underutilize microscopy and rely more on chest

radiographs for TB diagnosis (*17–19*). Thus, implementation of infection control measures might motivate the private healthcare sector to adopt the DOTS strategy, and implementation of the DOTS strategy may, in turn, enhance infection control.

Fourth, even though low-income countries have fewer resources, ignoring a potential hazard runs contrary to the principles of protecting human health, the cornerstone of health care in any country. Finally, the problem of controlling TB in hospitals is not a problem with TB alone but reflects a problem with infection control in general, which, if improved, could also prevent other infectious diseases (e.g., severe acute respiratory syndrome and avian influenza) that may be nosocomially transmitted. Thus, TB infection control programs can have secondary benefits. Ultimately, preventing outbreaks and protecting patients and staff are in the interests of healthcare facilities. TB infection control is a good starting point for such efforts.

In this article, we focus on India as a case study and review available studies on nosocomial TB, describe factors that facilitate nosocomial transmission, and consider the feasibility of various recommended TB infection control interventions. Finally, we outline critical questions that need to be studied to effectively address nosocomial TB. Although we focus on India, the issues we raise may be applicable to other high-prevalence, resource-limited countries.

Nosocomial TB in India

India has more TB patients than any other country (*20*) and accounts for one fifth of the world's incident TB cases (*21*); the reported incidence in 2003 was 168 per 100,000 (*20*). Every year, TB develops in nearly 2 million persons in India, and nearly 1 million cases are smear positive; an estimated 40% of the Indian population is latently infected with *M. tuberculosis* (*21*). India's Revised National TB Control Programme (RNTCP) now provides access to DOTS for >85% of the population (*21*). Countrywide coverage is anticipated in 2006 (*22*). This program is the fastest expanding DOTS program in the world and the largest in the world in terms of patients receiving initial treatment (*21*). Outside of the RNTCP, India has a large private health sector that is actively involved in providing TB care (*23*,*24*); almost half of patients with TB in India initially seek care from the private sector (*22*). Thus, because Indian healthcare workers see large numbers of TB patients and because large numbers of TB patients are hospitalized (*25*), the risk for nosocomial exposure is substantial.

Despite the prevalence of TB in India and the expected high probability of nosocomial transmission, little is known about nosocomial TB. In fact, until 2004, no studies on nosocomial TB in India had been published. Table 1 summarizes the results of recent studies on TB among healthcare workers from 3 large tertiary hospitals (*26–30*). These studies provide some data on the incidence of active TB (*28*,*29*), prevalence of latent TB infection (*26*), risk factors for active TB (*30*), and annual risk for latent TB infection among healthcare workers (*27*). In addition, another recent study documented person-to-person transmission of TB among hospitalized patients (*31*).

At a rural medical school hospital in Sevagram, Pai et al. performed the tuberculin skin test (TST) and a whole-blood interferon-γ release assay (IGRA) for 726 healthcare workers (*26*); 50% were positive by either TST or IGRA. Nearly 70% of the participants reported direct contact with sputum smear–positive TB patients. Exposure was particularly high among physicians in training, attending physicians, and nurses. Increasing age and duration of employment were risk factors for latent TB infection. Nurses, nursing students, orderlies, and laboratory staff had higher prevalence of latent infection (*26*). A repeat survey of 216 medical and nursing students in this cohort enabled estimation of the annual risk for latent infection by using TST and IGRA (*27*). When both tests were used, the annual risk for latent TB infection was estimated to be 5% (*27*). The estimated community-based annual risk for infection in India is 1.5% (*32*), so the excess risk of 3.5% may be attributable to nosocomial exposure.

At a tertiary care hospital in Chandigarh, Rao et al. estimated the incidence of active TB among resident physicians (*28*). Among residents already working in the hospital, TB developed in 9 (2%) of 470, for an incidence of 11.2 new cases per 1,000 person-years of exposure. Extrapulmonary disease developed in two thirds of the residents. Overall, this study showed a high rate of TB (predominantly extrapulmonary) among those who worked in medical subspecialties. However, most cases were identified by using clinical criteria, and few were bacteriologically confirmed.

In a retrospective review of healthcare workers who underwent anti-TB treatment in a tertiary care hospital in Vellore, Gopinath et al. identified 125 healthcare workers who had been treated for active TB between 1992 and 2001 (*29*). The annual incidence of pulmonary TB was 0.35–1.80 per 1,000 persons during this period. The annual incidence of extrapulmonary TB was 0.34–1.57 per 1,000. These rates may have been underestimated because only healthcare workers who underwent TB treatment were counted. In this hospital, a case-control study showed that low body mass index and employment in medical wards were risk factors for TB disease among healthcare workers (*30*).

In a molecular epidemiologic study at a TB hospital in Delhi, Bhanu et al. performed DNA fingerprinting on 83 *M. tuberculosis* isolates from patients in 2 adjacent wards (*31*). Of these 83 isolates, 8 strains were grouped into

Table 1. Recent studies on TB among HCWs in India*

Author, city, year	Setting	Population	Prevalence of latent TB	Incidence of latent TB	Incidence of active TB	Comments
Pai et al., Sevagram, 2005 (*26*)	Rural medical school	726 HCWs, including medical and nursing students (median age 22 y, 62% female) underwent both TST and IGRA	50% positive by TST or IGRA	NA	NA	Prevalence of LTBI was probably underestimated because of nonresponse among senior physicians
Pai et al., Sevagram, 2006 (*27*)	Rural medical school	216 medical and nursing students (median age 21 y) were tested with TST and IGRA; both tests were repeated after 18 mo to document conversions	22% positive by TST, 18% positive by IGRA	5% with TST and IGRA	NA	Annual risk for LTBI was probably underestimated because only students were included in the study
Rao et al., Chandigarh, 2004 (*28*)	Urban tertiary care hospital	701 resident doctors (470 [group 1] were already working at the hospital and 231 [group 2] were newly admitted to the institute); mean age 28 y in group 1 and 26 y in group 2, 81% male	NA	NA	TB developed in 4 of 231 newly admitted residents within 1 y of beginning work, incidence of 17 per 1,000; all except 1 had EPTB	High rate of active TB (mostly EPTB) among HCWs in medical specialties; few cases were bacteriologically confirmed
Gopinath et al., Vellore, 2004 (*29*)	Urban medical school	Retrospective survey to identify HCWs who had TB treatment between 1992 and 2001	NA	NA	125 HCWs underwent TB treatment between 1992 and 2001; 43% of all cases were EPTB, and 5% were MDRTB; incidence of pulmonary TB was 0.35–1.80 per 1,000; incidence of EPTB was 0.34–1.57 per 1,000	EPTB was common; largest number of cases was reported among nurses and nursing students
Mathew et al., Vellore, 2005 (*30*)	Urban medical school	101 HCWs who had had TB disease were compared with 101 randomly selected controls from the same hospital	NA	NA	NA	Body mass index <19 kg/m^2 and employment in medical wards were independent risk factors for TB disease

*TB, tuberculosis; HCWs, healthcare workers; TST, tuberculin skin test; IGRA, interferon-γ release assay; NA, not available; LTBI, latent TB infection; EPTB, extrapulmonary TB; MDRTB, multidrug-resistant TB.

3 clusters (identical fingerprints) by using IS*6110* restriction fragment length polymorphism and spoligotyping analyses. Within each cluster, epidemiologic data showed overlapping hospitalization periods, which raises the possibility of nosocomial transmission (*31*).

In summary, these studies suggest that nosocomial transmission of TB is a problem in India. The prevalence of latent TB infection and annual risk for TB infection appears to be high even among young healthcare workers. For example, in a hypothetical Indian hospital with 1,000 workers, ≈500 (50%) will likely have latent infection, and ≈25 (5%) of uninfected workers will be newly infected each year. The rate of active disease appears to be exceedingly high in subgroups such as interns, residents, and nurses. The incidences of TB disease and infection are higher than the national averages, which suggests an increased risk for acquiring TB in the hospital setting. For example, the estimated incidence of TB among residents was 10-fold higher than the incidence for the country (*28*).

The predominance of extrapulmonary (mostly pleural) disease among healthcare workers may indicate progression to disease from newly acquired primary infection rather than reactivation of latent TB. Molecular epidemiologic studies suggest that pleural TB is different from other forms of extrapulmonary TB and is associated with the highest fingerprint clustering rate of all forms of TB, which suggests that pleural TB may be an early manifestation of recent infection (*33*). Lastly, although this assumption is based on limited data, nosocomial transmission of TB among hospitalized patients may occur in urban hospitals.

Factors That May Facilitate Nosocomial Transmission

Several factors may facilitate nosocomial transmission in Indian hospitals, although their relative importance in facilitating transmission is unknown (Table 2). The overwhelming number of TB patients and repeated exposures to smear-positive TB patients are likely to be critical factors. The RNTCP alone starts treatment for >100,000 patients every month (*21*), and thousands more are managed in the private sector (*19,22–24*). Repeated exposure of trainees is particularly worrisome, given the lack of TB

Table 2. Factors that may facilitate nosocomial transmission of tuberculosis (TB) in hospitals in India

Area	Factor
Factors that increase risk for nosocomial exposure	Overwhelming numbers of TB patients and repeated exposure to smear-positive TB patients
	Unnecessary or prolonged hospitalization of smear-positive TB patients
	Delays in initiating anti-TB treatment for those with TB
	Poor adherence to treatment, use of suboptimal treatment regimens, and lack of adequate patient support to improve adherence
	Interruptions in supply of TB medications in healthcare facilities
Lack of effective infection-control procedures	Failure to recognize and isolate patients with active pulmonary TB
	Laboratory delays in identification of TB, and poor use of tests such as sputum microscopy to identify infectious TB cases
	Clustering patients with TB with susceptible and vulnerable patients (e.g., HIV-positive patients)
	Lack of HIV testing services and delayed recognition of TB in HIV-infected patients because of atypical presentation and low level of clinical suspicion
	Inadequate respiratory isolation facilities and engineering controls
	Overcrowded hospital wards and outpatient departments
	Poorly ventilated wards and rooms
	Lack of adequate sunlight in hospital wards and departments
	Lack of airborne infection isolation rooms
	Lack of personal protection equipment (e.g., respirators)
	Lack of screening programs to detect and treat TB among healthcare workers
	Lack of commitment on the part of hospitals to invest in infection control programs
	Lack of national guidelines on nosocomial TB tailored to the Indian healthcare environment
Gaps in knowledge and awareness	Lack of awareness about nosocomial TB transmission in healthcare settings in India
	Healthcare workers' belief that nosocomial infection is an occupational hazard that cannot be avoided
	Lack of educational programs on occupational safety and hygiene
	Poor patient education regarding cough etiquette and sputum disposal

infection control measures at most healthcare facilities. In India, students begin the undergraduate medical program at the age of 17 or 18 years. After an initial classroom-based program in basic sciences, they begin their clinical rotations during years 2 and 3. During this phase of their training, stress is placed on physical examination. Evaluation of the respiratory system, for example, is invariably included in licensure examinations. Because patients with cavitary TB are likely to exhibit signs during a lung exam, TB patients are considered excellent teaching material. Trainees spend considerable time eliciting physical signs in such patients, which results in repeated exposure to patients with infectious TB during trainees' first clinical rotations. This fact may explain the high incidence of infection among them (*27*).

Delays in diagnosis and initiation of treatment and failure to separate or isolate patients with smear-positive TB from other patients also contribute to transmission risk. Previous studies in India have shown that diagnostic delays are common, and private practitioners, in particular, tend to underuse sputum microscopy, thereby increasing the probability of missing infectious TB patients (*17*,*19*,*34*). Unnecessary or prolonged hospitalization of TB patients who could have been treated on an ambulatory basis might also contribute to high exposure levels in hospitals. A survey of TB hospitals in India showed that nearly 1 million patients sought treatment in 1999. Approximately 77% of these patients were reported to have undergone sputum examination, and one third of all patients had a diagnosis of TB (*25*). Approximately one third of the hospitals admitted every sputum smear–positive TB patient encountered at their institution.

Several factors might prolong infectiousness of TB patients and thereby facilitate nosocomial transmission. Poor adherence to treatment, lack of continuous drug supply, use of suboptimal treatment regimens, lack of adequate treatment support (e.g., direct observation of therapy [DOT]), and insufficient treatment duration have been reported, particularly in the private sector (*18*,*19*,*24*,*25*, *35*,*36*).

Few hospitals in India have established infection control procedures. Hospitals, especially publicly owned facilities, tend to be crowded, poorly ventilated, and have limited or no facilities for respiratory isolation. Most respiratory care procedures (including sputum collection) are routinely carried out in a general ward setting, rather than in respiratory isolation rooms. Further, few of these hospitals offer routine screening programs to detect and treat TB among healthcare workers.

Previous surveys have identified gaps in knowledge and awareness about TB in healthcare workers in India (*18*,*19*,*24*,*36*,*37*). A survey of 213 nurses in 2 hospitals in Delhi showed that only 67% reported *M. tuberculosis* as the causative organism, and only 22% reported sputum microscopy as the most appropriate way to diagnose TB (*37*). In another survey, only 12% of 204 private practitioners in Delhi reported ordering sputum smears for a patient with suspected TB. For treating TB, 187 physicians used

102 different regimens (*18*). Other surveys have reported similar findings in India (*17*,*19*,*24*,*35*,*36*). Finally, healthcare workers may believe that that they cannot avoid nosocomial infection, which results in resigned acceptance on their part. Since nearly half the Indian population is infected, healthcare workers do not view latent TB infection as a problem. Hence, latent infection is rarely treated, even in high-risk groups such as household contacts and HIV-infected patients (*38*,*39*).

Implementing TB Infection Control in India

Effective TB infection control in healthcare settings depends on early identification, isolating infected persons, and rapidly and effectively treating persons with TB (*2*,*4*,*5*). In all healthcare settings, a basic TB infection control program should be implemented, as recommended by WHO and other agencies (*2–5*,*7*). WHO also recommends developing an infection control plan, educating healthcare workers and patients, improving sputum collection practices, performing triage and evaluation of suspected TB patients in outpatient settings, and reducing exposure in the laboratory (*7*). In the United States, administrative controls (early detection, isolation, and treatment of patients with TB) have been the most effective components of TB infection control programs (*9*).

In India, of all the recommended interventions, implementing administrative controls is likely to be the most feasible and effective strategy. Controls include early detection of patients with infectious TB, isolating or at least segregating those with infectious pulmonary TB from other patients, and rapidly initiating anti-TB treatment, supported by measures to improve adherence (e.g., DOT).

Implementing many of the recommended engineering controls is not feasible in most healthcare facilities because of the high costs of such measures (e.g., negative-pressure isolation rooms). However, separation or segregation of smear-positive TB patients in private or semiprivate rooms or wards with simple mechanical exhaust ventilation (e.g., window fans) could be feasible in some settings, particularly in the private sector and well-funded public hospitals. These measures have been shown to be useful in terminating an outbreak of nosocomial tuberculosis (*9*). This intervention is particularly necessary at centers that manage patients with MDRTB; at such centers, patients with infectious TB must not be admitted to the same wards as patients with HIV infection.

Personal respiratory protection measures (e.g., N95 respirators) are probably not feasible because of the high cost. Respirators may be relatively costly to implement and of limited effectiveness in high-incidence, resource-limited settings. (*40*). The use of respirators may have a role in hospitals that manage MDRTB, but more successful and affordable measures include improving natural ventilation through open windows and sunlight. The efficacy of UV germicidal lights is being evaluated in other low-income countries, and results of such studies are needed to determine their value in reducing nosocomial transmission. In developing TB infection control programs, crucial issues are educating healthcare workers about nosocomial TB and measures that can help prevent such transmission, educating patients on cough procedures, and using simple surgical masks on patients with infectious TB (especially if they are not segregated) who are coughing.

Periodic testing of healthcare workers for latent TB and treating those with latent infections who are at high risk for progression to active TB might be feasible in selected settings, particularly among trainees and junior staff (who seem to be disproportionately affected). Screening for latent TB infection with newer, blood-based IGRAs may not be feasible in most settings at this time. Although IGRAs have some advantages over TST, including increased specificity and the ability to discriminate between infection with *M. tuberculosis* and *M. bovis* BCG, they have limited applicability in many resource-limited settings because of the high costs and the need for laboratory infrastructure (*26*,*41*). However, new data suggest that IGRAs hold promise for serial testing of healthcare workers and can overcome some of the limitations of serial tuberculin testing (*27*). A recent study from India showed that in a setting with intensive nosocomial exposure, healthcare workers had strong interferon-γ responses that persistently stayed elevated even after treatment for latent infection (*42*). Persistence of infection or reexposure might account for this phenomenon.

Evaluation of symptomatic healthcare workers for active TB is feasible and should be implemented routinely. In addition to the above measures, hospitals should make every effort to treat TB patients on an ambulatory basis (*25*). If hospitalization is required, every effort should be made to segregate potentially infectious patients from immunocompromised patients, rapidly diagnose and initiate treatment, and discharge patients promptly with DOT on an outpatient basis.

Lastly, efforts should be made to improve the quality of TB care in the private sector through better coordination between the RNTCP and the private sector (*22*). By improving TB diagnosis and treatment practices, smear-positive TB patients are more likely to receive rapid diagnosis and treatment, thereby directly and indirectly reducing the overall transmission in the community and in the nosocomial setting. Such public-private partnerships are currently ongoing in India (*22*), and these programs could address the issue of nosocomial TB.

Who should design and implement TB infection control programs in India? This is a complicated issue because of the variability of healthcare systems in India (e.g., public,

private, corporate, nongovernmental, and alternative medical systems). Further, the private sector in India is dominant, diverse, and largely unregulated (*22*). Although a few hospitals have received quality certifications (e.g., ISO 9000), no pressure is on healthcare facilities to get accredited; in fact, India has no national accrediting body. Also, a large proportion of Indians pay for health care with personal funds rather than health insurance.

Given these problems, we cannot envision a simple approach to implementing infection control programs in India. While technical guidance should come from international agencies such as WHO and the International Union Against Tuberculosis and Lung Disease, these guidelines need to be adapted to the Indian context by RNTCP. Ultimately, implementing adequate infection control measures is the responsibility of each healthcare facility. RNTCP may not have the regulatory authority to enforce implementation; however, by partnering with the private sector, RNTCP can improve the quality of case detection and treatment provided in the private sector, which can, by itself, improve infection control.

Call for Research and Action

Despite India's long and distinguished history of TB research, nosocomial TB has in large part not been addressed by researchers, at least until recently. Although a few studies have been published (*26–31*), many more are needed, as summarized in Table 3. A first step is to determine the prevalence of TB among healthcare workers and to evaluate risk factors for nosocomial transmission. In addition, we must assess the availability of resources in India to implement TB infection control measures and to assess what additional resources are needed in areas that have little or no TB infection control programs. India is a vast country with substantial regional variability in resources and expertise. Some healthcare facilities (e.g., private hospitals and medical schools) may have implemented control measures or may have the resources and skills needed to establish effective infection control programs.

After assessing the disease prevalence, risk factors, and resources, India must implement effective strategies to reduce nosocomial transmission. To intervene, we will need to know what interventions will and will not work in India. Trials are therefore needed to evaluate relatively simple, feasible interventions and their effectiveness in reducing nosocomial risk. The lessons learned in such trials will be applicable in other resource-limited settings.

In conclusion, healthcare workers are essential in the fight against TB, and their health needs to be protected. India, with its vast human and intellectual capital, nearly countrywide DOTS coverage, and a large, well-funded, successful national TB control program, is well placed to tackle this problem and set an example for other high-prevalence countries.

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Table 3. Research needs on nosocomial TB in India*

Area	Specific research questions
Epidemiology and prevalence of disease	What is the prevalence and incidence of latent and active TB among HCWs? Is TB in HCWs more prevalent than in the community?
Molecular epidemiology of transmission of *Mycobacterium tuberculosis* in healthcare settings	What is the likelihood of person-to-person transmission in healthcare settings? How common are nosocomial outbreaks?
Risk factors for exposure to *M. tuberculosis* and risk factors for acquiring LTBI and active disease	What are risk factors for acquiring TB? What are risk factors for patient-to-patient transmission? Why is extrapulmonary disease more common than pulmonary TB among HCWs?
Evaluation of newer diagnostic tools	What is the utility of IGRAs to estimate risk of infection among HCWs? Are IGRAs more accurate, feasible, and cost-effective than TSTs for serial testing of HCWs?
Interventions to reduce nosocomial transmission	What simple, feasible interventions can reduce nosocomial transmission? What is the cost-effectiveness of control programs, and what are long-term benefits to the health system? In HCWs with repeated exposure, what is the long-term efficacy of preventive therapy?
Social, operational, and behavioral issues	What operational and logistic factors increase risk for nosocomial exposure? How common are diagnostic and treatment delays, and how do they affect exposure levels? How does prolonged hospital stay affect risk for nosocomial transmission? How knowledgeable and aware of nosocomial TB are HCWs? What factors affect HCW adherence to interventions that might reduce transmission? How does TB among HCWs affect the healthcare workforce, and how does it affect healthcare delivery? What resources for TB infection control are available in India, and what type of variability exists across healthcare facilities in various states?

*TB, tuberculosis; HCW, healthcare worker; LTBI, latent TB infection; IGRA, interferon-γ release assay; TST, tuberculin skin test.

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Control of Avian Influenza in Poultry

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Avian influenza, listed by the World Organization for Animal Health (OIE), has become a disease of great importance for animal and human health. Several aspects of the disease lack scientific information, which has hampered the management of some recent crises. Millions of animals have died, and concern is growing over the loss of human lives and management of the pandemic potential. On the basis of data generated in recent outbreaks and in light of new OIE regulations and maintenance of animal welfare, we review the available control methods for avian influenza infections in poultry, from stamping out to prevention through emergency and prophylactic vaccination.

Avian influenza (AI), which emerged from the animal reservoir, represents one of the greatest recent concerns for public health. Compared with the number reported for the past 40 years, the number of outbreaks of AI in poultry has increased sharply during the past 5 years. The number of birds involved in AI outbreaks has increased 100-fold, from 23 million from 1959 through 1998 to >200 million from 1999 through 2005 (*1*). Since the late 1990s, AI infections have assumed a completely different profile in the veterinary and medical scientific communities. Some recent outbreaks have been minor, but other epidemics, such as the Italian 1999–2000, the Dutch 2003, the Canadian 2004, and the ongoing Eurasian, have been more serious. They have led to devastating consequences for the poultry industry, negative repercussions on public opinion, and, in some instances, created major human health issues, including the risk of generating a new pandemic virus for humans through an avian-human link.

Influenza viruses are segmented, negative-strand RNA viruses that are placed in the family *Orthomyxoviridae* in 3 genera: *Influenzavirus A*, *B*, and *C*. Influenza A viruses are the only type reported to cause natural infections of birds and are further divided into subtypes according to antigenic characteristics of the surface glycoproteins hemagglutinin (H) and neuraminidase (N). At present, 16 hemagglutinin subtypes (H1–H16) and 9 neuraminidase subtypes (N1–N9) have been identified. Each virus has one H and one N antigen, apparently in any combination; all subtypes and most possible combinations have been isolated from avian species.

Influenza A viruses that infect poultry can be divided into 2 distinct groups according to the severity of disease they cause. The most virulent viruses cause highly pathogenic avian influenza (HPAI), a systemic infection in which death rates for some susceptible species may be as high as 100%. These viruses have thus far been restricted to strains that belong to the H5 and H7 subtypes and have a multibasic cleavage site in the precursor of the hemagglutinin molecule. HPAI is a lethal infection in certain domestic birds (e.g., chickens and turkeys) and has a variable clinical effect (may or may not cause clinical signs and death) in domestic waterfowl and wild birds. The potential role of wild birds and waterfowl as reservoirs of infection by HPAI strains has been described for only the Asian HPAI virus H5N1. The ecologic and epidemiologic implications of this unprecedented situation are not predictable.

On the contrary, viruses that belong to all subtypes (H1–H16) that lack the multibasic cleavage site are perpetuated in nature in wild bird populations. Feral birds, particularly waterfowl, are the natural hosts for these viruses and are therefore considered an ever-present source of viruses. Since their introduction into domestic bird populations, these viruses have caused low-pathogenicity avian influenza (LPAI), a localized infection that results in mild disease, primarily respiratory disease, depression, and egg-production problems. Theories suggest that HPAI viruses emerge from H5 and H7 LPAI progenitors by mutation or recombination (*2*,*3*), although >1 mechanism is likely. This theory is supported by findings from phylogenetic studies of H7 subtype viruses, which indicate that HPAI viruses do not constitute a separate phylogenetic lineage or lineages but appear to arise from nonpathogenic strains (*4*,*5*); this indication is supported by the in vitro selection of mutants virulent for chickens from an avirulent H7 virus (*6*).

Such mutation probably occurs after the viruses have moved from their natural wild-bird host to poultry.

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However, the mutation to virulence is unpredictable and may occur very soon after the virus is introduced to poultry or after the LPAI virus has circulated in domestic birds for several months. This hypothesis is strongly supported by a recent study of Munster et al. (*7*), who showed that minor genetic and antigenic diversity exists between H5 and H7 LPAI viruses found in wild birds and those that caused HPAI outbreaks in domestic poultry in Europe. The scientific evidence collected in recent years leads to the conclusion that not only must HPAI viruses be controlled in domestic populations, but LPAI viruses of the H5 and H7 subtypes should also be controlled because they represent HPAI precursors.

Prevention of Avian Influenza

From December 1999 through April 2003, >50 million birds died or were depopulated after HPAI infection in the European Union (*1*), causing severe economic losses to the private and public sectors. These losses suggest that the strategies and control measures used to combat the disease need improvement, from disease control and animal welfare perspectives.

AI viruses are introduced to domestic poultry primarily through direct or indirect contact with infected birds. Transmission may occur through movement of infected poultry; movement of contaminated equipment, fomites, or vehicles; and exposure to contaminated infectious organic material. Airborne transmission over long distances between farms has not yet been demonstrated. For these reasons, if biosecurity measures are implemented at the farm level, AI infections can be prevented.

Outbreaks that involve large numbers of animals are characterized by the penetration of infection into the commercial circuit; that is, industrially reared poultry and all other poultry that is traded, including those from semi-intensive and backyard farms. Biosecurity (encompassing bioexclusion and biocontainment) represents the first and most important means of prevention. If biosecurity measures of a high standard are implemented and maintained, they create a firewall against infection penetration and perpetuation in the industrial circuit. However, breaches in biosecurity systems do occur. On one hand, the occurrence and extent of the breach should be evaluated and corrective measures should follow; on the other, they indicate the need to establish early warning systems and additional control tools for AI.

General Aspects of Vaccination

Until recently, AI infections caused by viruses of the H5 and H7 subtype occurred rarely, and vaccination was not considered because stamping out was the recommended control option. Primarily for this reason, vaccinology for AI has not grown at the same rate as for other infectious diseases of animals. Data are being generated from experimental and field research in AI vaccinology, but the rather complex task of vaccinating poultry in different farming and ecologic environments still has areas of uncertainty.

Guidelines on disease prevention and control have been issued as joint recommendations of the World Organization for Animal Health (OIE), the Food and Agriculture Organization (FAO), and the World Health Organization (*8*). These recommendations, however, need to be put into practice in a variety of different field situations; the applicability of 1 system rather than another in a given situation must be evaluated, weighing the benefits of a successful result against the drawbacks of failure.

Vaccination can be a powerful tool to support eradication programs if used in conjunction with other control methods. Vaccination has been shown to increase resistance to field challenge, reduce shedding levels in vaccinated birds, and reduce transmission (*9,10*). All these effects of vaccination contribute to controlling AI; however, experience has shown that, to be successful in controlling and ultimately in eradicating the infection, vaccination programs must be part of a wider control strategy that includes biosecurity and monitoring the evolution of infection.

To eradicate AI, the vaccination system must allow the detection of field exposure in a vaccinated flock, which can be achieved by using conventional inactivated vaccines and recombinant vector vaccines. Conventional inactivated vaccines that contain the same viral subtype as the field virus enable detection of field exposure when unvaccinated sentinels left in the flock are tested regularly. This system is applicable in the field but is rather impracticable, especially for the identification of sentinel birds in premises that contain floor-raised birds. A more encouraging system, based on the detection of anti-NS1 antibodies, has been recently developed and can be used with all inactivated vaccines, provided they have the same hemagglutinin subtype as the field virus (*11*). This system is based on the fact that the NS1 protein is synthesized only during active viral replication and, therefore, is rarely present in inactivated vaccines. Birds vaccinated with such vaccines will develop antibodies to NS1 only after field exposure. Full and field testing of this system under different circumstances are still in progress (*11,12*), and results should be available before this system is recommended.

To date, the only system that enables detection of field exposure in a vaccinated population and that has resulted in eradication is based on heterologous vaccination and known as "DIVA" (differentiating infected from vaccinated animals). This system was developed to support the eradication programs in the presence of several introductions of LPAI viruses of the H7 subtype (*1,9*). Briefly, a vaccine is used that contains a virus possessing the same hemagglutinin, but a different neuraminidase, as the field

virus. This vaccination strategy enables detection of antibodies to the neuraminidase antigen of the field virus. For example, a vaccine containing an H7N3 virus can be used against a field virus of the H7N1 subtype. Antibodies to H7 are cross-protective, thus ensuring clinical protection, increased resistance to challenge, and reduction of shedding, while antibodies to the neuraminidase of the field virus (in this case N1) can be used as a natural marker of infection. Experimental data on the quantification of the vaccination effect on transmission within a flock indicate that the reproduction ratio can be reduced to <1 by 1 week after vaccination (*10*). Such a reproduction ratio indicates minor rather than major spread of infection. In simple terms, such vaccination interventions will substantially reduce (although not prevent) secondary outbreaks, depending on the immune status of contact birds and flock.

Promising results have also been obtained with vaccines generated by reverse genetics (*13*). These vaccines are expected to perform like conventional inactivated vaccines; however, data are not yet available as to their efficacy under field conditions. Recombinant fowlpox vaccines that express the hemagglutinin protein of the field virus have also been reported to be efficacious for reducing shedding levels and providing clinical protection (*14*). They enable the detection of field exposure because vaccinated unexposed animals do not have antibodies to any of the other viral proteins. Any test developed to detect antibodies to the nucleoprotein, matrix, NS1, or neuraminidase of the field virus can be used to identify field-exposed birds in a vaccinated population. However, the performance of these vaccines in relation to the immune status of the host to the vector virus is unclear (*15*). Recent encouraging studies indicate that vaccination of day-old chicks with maternal antibodies against fowlpox has been successful. Data are lacking on the performances of such vaccines in a population that has been field exposed to fowlpox. Another aspect that must be carefully considered is the host. These vaccines are likely to induce protective immunity only in birds that are susceptible to infection with the vector virus.

Regardless of the vaccine and companion test used, mapping occurrence of infection within the vaccinated population is imperative, primarily to monitor the evolution of infection and to appropriately manage field-exposed flocks. Field exposure represents a means by which infectious virus may continue to circulate in the immune population; for this reason, vaccination can be considered as only part of a control strategy based on biosecurity, monitoring, approved marketing procedures, and stamping out. An inappropriately managed vaccination campaign will likely result in the virus becoming endemic.

Inadequate biosecurity or vaccination practices can lead to transmission between flocks and selection of variants that exhibit antigenic drift. Antigenic drift of H5N2 viruses belonging to the Mexico lineage, resulting in lower identity (less similarity) to the vaccine strain, has been described (*16*). Extensive use of vaccine in Mexico has resulted in the emergence of antigenic variants that escape the immune response induced by the vaccine. This occurrence is similar to antigenic drift that typically occurs in animals with a long lifespan (pigs and horses) that are routinely vaccinated and in human beings. Mexico has been vaccinating poultry since the HPAI outbreak in 1994 without applying the DIVA principle. Although no HPAI virus has been reported since the implementation of the vaccination campaign, LPAI viruses continue to circulate. Conversely, a similar approach in Pakistan after the HPAI H7N3 outbreaks in 1995 resulted in the isolation of HPAI H7N3 virus ≈10 years later, in 2004 (*17*).

The international scientific community is debating how vaccination of poultry would affect human health. On one hand, vaccinated birds shed less virus; on the other, they do not show any clinical signs of disease and could therefore act as silent carriers. Several factors contribute to the development of infection in humans: insufficient hygienic standards, the characteristics of the strain, and presence of viral dose sufficient to infect a human being. The possibility that vaccinated poultry may not shed enough virus to infect a human being is substantiated by recent field evidence. With reference to the H5N1 crisis, several countries are using vaccination to support control efforts. Vietnam implemented a nationwide vaccination campaign, which was completed in early 2006. The campaign's main achievement is that despite 61 cases of human infection between January and November 2005, no human cases of AI have been reported in Vietnam after December 2005 (*18*).

Emergency Vaccination

Recent outbreaks in developed countries, notwithstanding their efficient veterinary infrastructures and modern diagnostic systems, have resulted in the culling of millions of birds. Since the year 2000, AI epidemics in areas densely populated with poultry have resulted in 13 million dead birds in Italy in 1999–2000 (H7N1), 5 million dead birds in the United States in 2002 (H7N2), 30 million in the Netherlands in 2003, and 17 million in Canada in 2004. For each of these episodes, biosecurity measures implemented at the farm level were insufficient to prevent massive spread of AI.

Emergency vaccination for AI has become an acceptable tool, in conjunction with other measures, for combating the spread of AI. Using emergency vaccination to reduce the transmission rate could provide an alternative to preemptive culling to reduce the susceptibility of healthy flocks at risk. The effectiveness of such a program depends on variables such as the density of poultry flocks in the

area, level of biosecurity and its integration into the industry, characteristics of the virus strain involved, and practical and logistical issues such as vaccine availability and adequate and speedy administration. For this reason, contingency plans that include decision-making patterns under different scenarios should be formulated.

Pivotal work on emergency vaccination has been done in Italy. Application of the DIVA strategy has resulted in the approval of the use of vaccination as an additional tool for the eradication of 2 epidemics of LPAI (H7N1 and H7N3) without massive preemptive killing of animals. Vaccination complemented restriction measures already in place and was integrated into an intensive monitoring program that identified viral circulation in the area (*9*) and culled infected birds. In 2000, heterologous vaccination against an H7 virus was used for the first time in the field as a natural marker vaccine. Subsequently, a DIVA strategy was used by Hong Kong to prevent the introduction of H5N1 into its territories (*19*).

Although use of a DIVA system enabled international trade of poultry products to continue (*9*,*20*), vaccination for AI is a new concept, which several countries are reluctant to even consider. Government authorities ultimately decide whether vaccination should be used in a given country; their reluctance is probably driven by legislative and scientific uncertainties, coupled with doubts about how this practice will be used in the field and other considerations such as exit strategy. With reference to trade implications, a new chapter of the OIE Terrestrial Animal Health Code on AI (*21*) enables the continuation of trade in presence of vaccination if the exporting country is able to produce surveillance and other data that confirm that notifiable avian influenza is not present in the compartment from which the exports come. This chapter is the result of extensive work by OIE experts and the OIE Central Bureau on the issue of reducing the effect of animal diseases through the use of vaccination and is contained in a recommendation document issued as a result of an international conference held in Buenos Aires (April 14–17, 2004) that strongly supports the use of vaccines for diseases on list A (*22*).

Prophylactic Vaccination

Prophylactic vaccination for viruses of the H5 and H7 subtypes is a completely innovative concept, primarily because only recently have cost-effective situations been identified. Prophylactic vaccination should generate a level of protective immunity in the target population; the immune response may be boosted if a field virus is introduced. Prophylactic vaccination should increase the resistance of birds and, in the case of virus introduction, reduce levels of viral shedding, provided the same levels of biosecurity are maintained. It should be perceived as a tool to maximize biosecurity measures when risk of exposure is high. Ideally, it should prevent the index case. Alternatively, it should reduce the number of secondary outbreaks, thus minimizing the negative effects on animal welfare and potential economic losses in areas where the density of the poultry population would otherwise result in uncontrollable spread without preemptive culling.

Prophylactic vaccination should be considered only when circumstantial evidence indicates that a given area is at risk. Risk for infection may be divided into 2 categories: 1) high risk for infection with either H5 or H7 subtype (e.g., from migratory birds), and 2) risk for infection with a known subtype (e.g., H7N2 in live bird markets in the United States, countries with high exposure to H5N1). For the first category, a bivalent (H5 and H7) vaccination program could be implemented. Italy has recently implemented such a program in the densely populated poultry area at risk for infection (*23*). For the second category, a monovalent (H5 or H7) program would be sufficient.

The choice of vaccine is crucial to the outcome of prophylactic vaccination campaigns. Ideally, vaccines that enable detection of field exposure with any AI virus should be used. Such candidates would be vaccines that enable the identification of field-exposed flocks through the detection of antibodies to an antigen that is common to all type A influenza viruses such as NP, M, or NS1. Such a strategy would detect the introduction of any subtype of AI.

The DIVA system, which uses heterologous neuraminidase, has some limitations in its application for prophylaxis or in situations with risk for introduction of multiple AI subtypes because the system was originally developed to fight a known subtype of AI. The main problem is that the virus against which vaccination is directed must have a different N subtype than the virus present in the vaccine, which, for prophylactic vaccination, is impossible to establish beforehand. An approach to resolving this difficulty is to use seed vaccine strains of the H5 and H7 subtypes that are exhibiting rare neuraminidase subtypes such as N5 or N8. This selection criterion of vaccine strains will greatly reduce the chance that an AI virus of a similar N subtype is introduced. In any case, for surveillance purposes, unvaccinated sentinels should be present in the flock.

Prophylactic vaccination should be continued as long as risk for infection exists. It can be used in a targeted manner for limited periods of time, which requires a detailed exit strategy.

Conclusions

The scientific veterinary community must control AI infections in poultry for several reasons: to manage the pandemic potential, to preserve profitability of the poultry industry, and to guarantee food security to developing

countries. Although biosecurity is recognized as an excellent means of preventing infection, in certain situations the biosecurity standards necessary to prevent infection are difficult to sustain. Vaccination is a potentially powerful tool for supporting eradication programs by increasing the resistance of birds to field challenge and by reducing the amount and duration of virus shed in the environment. Vaccination strategies that encompass monitoring of infection in the field are crucial to the success of such efforts.

Timely information is needed about the efficacy of vaccination in a variety of different avian species, bearing in mind the diverse farming systems used in developed and developing countries. The outcome of such efforts should be made available to the international community because decision makers lack enough information to make educated choices. An enormous effort is required from national governments and funding bodies to make resources available to research programs to develop improved control measures that can be applied under different local conditions. To maximize the global effort to combat this disease, developing and sustaining transversal research programs on AI control, which encompass veterinary and agricultural science, are imperative.

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Risk Factors for Buruli Ulcer, Benin

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We identified risk factors for Buruli ulcer (BU) in Benin in an unmatched case-control study at the Centre Sanitaire et Nutritionnel Gbemoten in southern Benin. A total of 2,399 persons admitted from 1997 through 2003 and 1,444 unmatched patients with other conditions in 2002 were recruited. Adjusted odds ratios were determined for age, sex, place of residence, *Mycobacterium bovis* BCG vaccination at birth, type of water for domestic use, and occupation. Children <15 years of age and adults >49 years of age had a higher risk for BU. Use of unprotected water from swamps was associated with increased risk for BU; this association was strongest in adults >49 years of age. Sex was not a risk factor for BU. Our data showed that BU was mainly associated with age, place of residence, and water sources in all age groups. Risk for BU was higher in BCG-vaccinated patients ≥5 years of age.

The World Health Organization (WHO) defines Buruli ulcer (BU) as "an infectious disease involving the skin, caused by *Mycobacterium ulcerans*, characterized by a painless nodule, papule, plaque or edema, evolving into a painless ulcer with undermined edges, often leading to invalidating sequelae. Sometimes bones are destroyed." (*1*). After tuberculosis and leprosy, BU is the third most common mycobacterial disease (*1*). Incidences have increased recently, especially in West Africa (*2*–*6*). In 1997, WHO recognized BU as an emerging public health problem.

Classically, BU is a disease of rural areas and associated with wetlands. Endemic foci exist in tropical Africa, the Americas, Australia, and Asia (*1*,*7*,*8*). Epidemiologic observations concentrate on descriptive information or identification of new foci; however, few case-control studies have been published (*2*,*3*,*9*). Our objective was to assess sociodemographic variables and selected environmental factors associated with BU in Benin.

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Patients and Methods

Setting

Benin is a West African country with 6,752,569 inhabitants: 51% are female (Third General Census, 2002. unpub. data). The population is young: 48% are <15 years old. Benin was formerly divided into 6 regions (Atlantique, Mono, Borgou, Zou, Oueme, and Atacora); however, each region is now divided into 2 regions (Atlantique/Littoral, Mono/Couffo, Borgou/Alibori, Zou/Colline, Oueme/Plateau, and Atacora/Donga).

The Centre Sanitaire et Nutritionnel Gbemoten (CSNG) in Zou, Benin, is the regional referral center for BU treatment. From 1989 through 2003, CSNG treated >4,000 BU patients. This study included the period 1997–2003. The population of Zou in 2001 was 1,112,943.

Sample Size

Endemic foci of BU are associated with stagnant bodies of water (ponds, backwaters, and swamps). Data from the Benin Demographic and Health Survey (DHS) 2001 show that 34.5% of the Zou community drink unprotected water (rain water, unprotected well, surface water) (*10*). The sample size calculated with EpiInfo (Centers for Disease Control and Prevention, Atlanta, GA, USA) was 147 cases and 147 hospital controls (control-case ratio 1, odds ratio [OR] ≥ 2, proportion of exposition to unprotected water in controls 34.5%, power 80% [$1 - \beta$], and 95% confidence level $\alpha = 0.05$).

Since we had a large amount of data at our disposal, we analyzed the entire group of case-patients and all controls recruited during 6 months in 2002. The duration of enrollment of controls was limited to 6 months because of problems of availability of the healthcare staff.

Case-Patient Recruitment

All patients with suspected BU were examined by a physician (C.Z.) and the nurse (J.A.) in charge of the CNSG. Historic and clinical data were recorded on standard forms. One or more of the following features

suggested by WHO were used to diagnose BU (*1*): typical nodular, indurated plaque or edematous lesion; ≥1 painless chronic ulcers with undermined edges or a depressed scar; swelling over a painful joint, suggesting bone involvement; slowly developing lesion (weeks or months); no fever or regional lymphadenopathy; and residence or travel in a zone endemic for BU.

CSNG admitted 2,468 suspected BU patients from 1997 through 2003. Sixty-nine patients were excluded: 56 with recurrent cases and 13 with another definitive diagnosis; 2,399 patients with BU were enrolled in the study.

Hospital Control Recruitment

In 2002, controls were recruited among patients seen at CSNG for conditions other than BU: 21% had malaria, 6.0% had bronchitis, 5.6% had rheumatism, and 5.5% had parasitosis. The frequency of all other conditions was <5%.

CSNG had 2 types of clinics: 1 for persons <5 years old and 1 for all others. Both clinics interviewed and examined people 3 times a week. For those ≥5 years old (group 1), the head nurse interviewed all patients with a disease other than BU. For those <5 years old (group 2), the parents or a guardian of 5 subjects were interviewed from each clinic. This method generated an unmatched control group of 1,444 persons.

Ethics

The Ethical Committee of Benin approved this study. All persons or their mothers or guardians received information about the disease and its treatment. All BU cases and controls received treatment. Verbal consent was obtained from participants or their parents or guardians.

Data Collection

Data were available from case files; information on the same variables was collected from controls. Data were then analyzed to identify potential risk factors for BU. All data on age, sex, region of residence, occupational activity, *Mycobacterium bovis* BCG scar status, and type of domestic water used were recorded during the clinical examination.

Water sources were categorized into 4 groups: pumped water, river, mixed sources (pumped and river), and swamps. The 4 groups were classified into 2 groups: protected water (pumped) and unprotected water (all other sources). Residences were categorized as Zou, Oueme, and other.

A total of 525 children were in group 1: 339 BU cases and 186 controls. Complete data, except water sources, were available for 339 persons (case-patients and controls). These persons were analyzed by logistic regression. When water source was entered into the model, this number decreased to 246. Age was divided into 3 groups: <1 year, 1–2 years, and 3–4 years. Occupation was not introduced into the model for children <5 years of age.

Group 2 had 3,252 persons: 2,032 BU patients and 1,220 controls. Because data were missing for 930 persons (775 BU patients and 155 controls), 2,322 persons were analyzed by logistic regression. This number was reduced to 1,775 and 1,332 persons, respectively, when water sources and water sources or activities were introduced into the model. Age was divided into 4 groups based on the ORs shown in Table 1: 5–14 years, 15–29 years, 30–49 years, and ≥50 years. Occupation was grouped as follows: attending or not attending school (for school-age persons), farmer, housewife, and other (e.g., salesperson, tailor, hairdresser, or teacher).

Data Analysis

Data were analyzed with SPSS version 9.05 (SPSS Inc., Chicago, IL, USA) and EpiInfo version 6.02. Because of the selection method of controls in the clinic for children, control children <5 years old were underrepresented, and analysis was performed separately for these children. Crude ORs and 95% confidence intervals (CIs) were calculated to evaluate the association between BU and various factors. Contingency tables were analyzed with the Pearson χ^2 test.

A backward elimination procedure, based on likelihood ratio, was used to select variables to include in logistic regression models. Because age and occupation were correlated (Spearman $\rho = 0.626$), we introduced either age or occupation into the logistic models. Some interactions were tested in the logistic model including age with the likelihood ratio test. Interaction between age and water source appeared significant; thus, separate logistic models were established for each age category. Because sex and occupation were virtually redundant (e.g., female and housewife), models including occupation were established separately for each sex. Adjusted ORs and 95% CIs were derived from the final logistic models. For these models, goodness of fit was assessed with the Hosmer and Lemeshow test.

Results

Group 1

Table 2 shows results of univariate analysis for group 1 (crude ORs and 95% CIs). Age, place of residence, and water sources were associated with BU. Sex was not associated with BU. The risk for BU was ≈3× higher in children without a BCG scar. BU was associated with having a BCG scar ($p = 0.047$). Risk for BU was particularly high when the water source was a swamp compared with pumped water (OR 47.59, 95% CI 13.76–164.61).

Table 1. Distribution of cases of Buruli ulcer and controls by age, Centre Sanitaire et Nutritionnel Gbemoten, Zou, Benin*

Age group, y	Cases (%)	Controls (%)	Total	Crude OR (95% CI)	p value
5–9	432 (21.3)	38 (3.1)	470	6.69 (2.86–16.62)	<0.001
10–14	405 (19.9)	59 (4.8)	464	4.4 (1.77–9.24)	0.002
15–19	223 (11.0)	117 (9.6)	340	1.12 (0.5–2.53)	NS
20–24	163 (8.0)	140 (11.5)	303	0.68 (0.3–1.54)	NS
25–29	149 (7.3)	157 (12.9)	306	0.56 (0.25–1.26)	NS
30–34	84 (4.1)	140 (11.5)	224	0.35 (0.15–0.81)	0.010
35–39	72 (3.5)	136 (11.1)	208	0.31 (0.14–0.72)	0.0046
40–44	44 (2.2)	98 (8.0)	142	0.26 (0.11–0.62)	0.002
45–49	53 (2.6)	81 (6.6)	134	0.38 (0.16–0.9)	0.025
50–54	68 (3.3)	60 (4.9)	128	0.67 (0.28–1.57)	NS
55–59	66 (3.2)	45 (3.7)	111	0.86 (0.36–2.06)	NS
60–64	105 (5.2)	52 (4.3)	157	1.19 (0.51–2.78)	NS
65–69	65 (3.2)	38 (3.1)	103	1.01 (0.42–2.42)	NS
70–74	50 (2.5)	32 (2.6)	82	0.92 (0.37–2.26)	NS
75–79	36 (1.8)	17 (1.4)	53	1.25 (0.47–3.29)	NS
≥80	17 (0.8)	10 (0.8)	27	1	
Subtotal	2,032	1,220	3,252		
Missing	367	224	591		
Total	2,399	1,444	3,843		

*OR, odds ratio; CI, confidence interval; NS, not significant.

Table 3 shows results of multivariate analysis for group 1. In this model, age, place of residence, and water sources were associated with BU. The risk for BU was high in children 3–4 years old (OR 6.74, 95% CI 2.67–17.03), those living in Oueme (OR 2.78, 95% CI 1.35–5.70) or other places (OR 9.74, 95% CI 2.27–41.76), and those using unprotected water (OR 2.27, 95% CI 1.22–4.22). Sex and a BCG scar were not associated with BU.

Group 2

Table 1 shows distribution of case-patients and controls by age group. The age groups used (5–14, 15–29, 30–49, and ≥50 years) were based on the crude ORs found. The 30- to 49-year age group had the lowest risk for BU.

Table 4 shows results of univariate analyses for persons ≥5 years old. All variables were associated with BU infection. Risk for BU was highest in persons using mixed sources of water (OR 16.72, 95% CI 10.63–26.31). The risk for BU in those using river water was similar to that of

Table 2. Univariate analysis of risk factors for Buruli ulcer in children <5 years of age, Centre Sanitaire et Nutritionnel Gbemoten, Zou, Benin*

Variable	Cases (%) (n = 339)	Controls (%) (n = 186)	Crude OR	95% CI	p value
Age, y					<0.001
<1	31 (9.1)	44 (23.7)	1		
1–2	154 (45.4)	103 (55.4)	2.12	1.26–3.58	
3–4	154 (45.4)	39 (21.0)	5.60	3.14–10.00	
Sex					NS
Female	169 (50.6)	85 (46.4)	1		
Male	165 (49.4)	98 (53.6)	0.85	0.59–1.22	
Mycobacterium bovis BCG scar					0.047
Present	158 (92.4)	170 (97.1)	1		
Absent	13 (7.6)	5 (2.9)	2.80	0.98–8.03	
Region					0.001
Zou	238 (70.2)	152 (81.7)	1		
Oueme	60 (17.7)	29 (15.6)	1.32	0.81–2.15	
Other	41 (12.1)	5 (2.7)	5.24	2.02–13.55	
Water sources					<0.001
Protected (pump)	29 (30.2)	92 (54.8)	1		
Unprotected (combined types)	67 (69.8)	76 (45.2)	2.80	1.59–4.93	
River	7 (7.3)	44 (26.2)	0.50	0.21–1.24	
Unprotected (mixed)	15 (15.6)	29 (17.3)	1.64	0.77–3.47	
Swamp	45 (46.9)	3 (1.8)	47.59	13.76–164.61	

*OR, odds ratio; CI, confidence interval; NS, not significant.

Table 3. Multivariate analysis of risk factors for Buruli ulcer in 246 children (87 case-patients and 159 controls) <5 years of age, Centre Sanitaire et Nutritionnel Gbemoten, Zou, Benin*

Variable	Adjusted OR†	95% CI	p value
Age, y			<0.001
<1	1		
1–2	1.58	0.62–3.97	
3–4	6.74	2.67–17.03	
Region			<0.001
Zou	1		
Oueme	2.78	1.35–5.70	
Other	9.74	2.27–41.76	
Water sources			0.010
Protected	1		
Unprotected	2.27	1.22–4.22	

*OR, odds ratio; CI, confidence interval.
†Adjusted for effects of all variables included in the model. Nonsignificant variables were sex and *Mycobacterium bovis* BCG scar.

those using swamp water. When water sources were classified as protected and unprotected water, the OR was 5.15 (95% CI 4.22–6.28) for those using unprotected water.

In the 5–14-year age group, no difference concerning schooling was observed between case-patients and controls. In those >14 years old, more controls were housewives than were case-patients (28.5% vs. 2.6%), and more case-patients were farmers than were controls (49.4% vs. 29.0%).

Univariate analyses by sex and occupation showed that in males, being school age and being a farmer produced the higher risk for BU (data not shown). Young boys (independent of school attendance) had the greatest risk. In females, school-age girls and farmers had similar risks for BU, but housewives had the lowest risk (data not shown).

Table 5 shows results of logistic regression analyses of risk factors for BU in persons ≥5 years old. Occupation was excluded because it correlated with age. The model included age, BCG scar, place of residence, and water sources but excluded sex (not significant). When adjusted for other variables, children 5–14 years old had a higher risk for BU (OR 11.64, 95% CI 8.01–16.91). The OR (95% CI 3.04–6.27) for BU was 4.36 in those ≥50 years old. Risk for BU was associated with exposure to unprotected water sources (OR 4.62, 95% CI 3.61–5.90). Absence of a BCG scar decreased the risk for BU (OR 0.40, 95% CI 0.31–0.52). Living a greater distance from CSNG was strongly associated with an increased risk for BU (OR 13.02, 95% CI 8.03–21.11).

Logistic regression showed a strong interaction between age and water source. Thus, a logistic model was produced for each age group. Adjusted ORs and 95% CIs derived from models in the different age groups showed that absence of a BCG scar decreased the risk for BU in all age groups (data not shown). Risk for BU associated with residence of patients was reduced in those ≥50 years old. BU patients were 1.94× more likely than controls to use unprotected water (OR 1.94, 95% CI 1.20–3.12), and ORs were at least twice those in other age groups (data not shown).

Final results of multivariate analyses by sex and occupation in patients ≥5 years old showed that the risk for BU was similar in both sexes for the following factors: BCG

Table 4. Univariate analysis of risk factors for Buruli ulcer in persons ≥5 years of age, Centre Sanitaire et Nutritionnel Gbemoten, Zou, Benin*

Variable	Cases (%) (n = 2,032)	Controls (%) (n = 1,220)	Crude OR	95% CI	p value
Age, y					<0.001
5–14	837 (41.2)	97 (8.0)	15.52	11.96–20.13	
15–29	535 (26.3)	414 (33.9)	2.32	1.90–2.84	
30–49	253 (12.5)	455 (37.3)	1		
≥50	407 (20.0)	254 (20.8)	2.88	2.31–3.59	
Sex					<0.001
Female	986 (49.2)	675 (57.0)	1		
Male	1,017 (50.8)	510 (43.0)	1.36	1.18–1.57	
Mycobacterium bovis BCG scar					<0.001
Present	969 (75.6)	610 (55.7)	1		
Absent	313 (24.4)	486 (44.3)	0.41	0.34–0.48	
Region					<0.001
Zou	1,110 (54.6)	883 (72.4)	1		
Oueme	575 (28.3)	298 (24.4)	1.53	1.30–1.81	
Other	347 (17.1)	39 (3.2)	7.08	5.02–9.97	
Water sources					<0.001
Protected (pump)	296 (36.2)	867 (74.5)	1		
Unprotected (combined types)	522 (63.8)	297 (25.5)	5.15	4.22–6.28	
River	75 (9.2)	47 (4.1)	4.67	3.17–6.89	
Unprotected (mixed)	137 (16.7)	24 (2.1)	16.72	10.63–26.31	
Swamp	310 (37.9)	226 (19.4)	4.02	3.24–4.99	

*OR, odds ratio; CI, confidence interval.

Table 5. Logistic regression analysis of risk factors for Buruli ulcer in 1,775 persons (754 case-patients and 1,021 controls) ≥5 years of age, Centre Sanitaire et Nutritionnel Gbemoten, Zou, Benin*

Variable	Adjusted OR†	95% CI	p value
Age, y			<0.001
5–14	11.64	8.01–16.91	
15–29	2.21	1.60–3.05	
30–49	1		
≥50	4.36	3.04–6.27	
Mycobacterium bovis BCG scar			<0.001
Present	1		
Absent	0.40	0.31–0.52	
Region			<0.001
Zou	1		
Oueme	4.02	3.09–5.24	
Other	13.02	8.03–21.11	
Water sources			<0.001
Protected	1		
Unprotected	4.62	3.61–5.90	

*OR, odds ratio; CI, confidence interval.
†Adjusted for effects of all variables included in the model. The nonsignificant variable was sex.

scar, place of residence, and water sources (data not shown). Risk for BU in boys was strongly associated with school age (OR 4.63, 95% CI 2.63–8.15). This risk was lower in girls (OR 2.75, 95% CI 1.59–4.76). Risk in female farmers was strongly increased (OR 3.51, 95% CI 1.97–6.26). Housewives showed a strong decreased risk for BU (OR 0.06, 95% CI 0.03–0.13).

Discussion

This is the first large case-control study to describe sociodemographic and environmental factors associated with BU. Our data show that BU was associated with age, place of residence, and water sources for all persons, and that BU was associated with a BCG scar in persons ≥5 years old.

In accordance with previous studies (*1–3*,*11*), the risk for BU was higher in children <15 years old. Children 3–4 years old had an increased risk for BU if they used unprotected water and lived in areas endemic for BU. That clothing provides protection against BU has already been reported in Côte d'Ivoire (*3*). In Africa, infants ≤1 year of age are often protected by clothes, bonnets, and booties. At ≈1–2 years of age, children begin to walk and play in dirt near their home, and those 3–4 years of age are more independent and roam freely in the environment, usually scantily clothed, which increases exposure to a contaminated environment. Use of unprotected water increases BU risk (OR >40), especially in children <5 years old. These associations have been previously reported (*9*,*12*,*13*). Molecular studies showed *M. ulcerans* in water, mud, fish, aquatic insects, and snails from swamps in regions endemic for BU (*14–18*). Material from swamps, ponds, or river regions may contaminate skin surfaces with *M. ulcerans*, which can result in introduction of the causative agent into skin when it is broken by trauma or insect bites (*15*,*19*).

Children 5–14 years old and persons >49 years old were at higher risk for BU. Their attire and contact with environmental sources of *M. ulcerans* may be a relevant factor. Elderly persons through repeated episodes of exposure to *M. ulcerans* may acquire latent infections that are reactivated by age-related immunosuppression. Results from the logistic model in the ≥50-year age group support this concept. In this age group, risk for BU showed a lower correlation with unprotected water than in other age groups.

Although adults 15–49 years old are frequently exposed to wetlands, their risk for BU was lower, which suggests acquired resistance to the disease. We speculate that this resistance may be related to acquired specific immunity or to cross-immunity from other mycobacterioses (*20*).

We previously reported that men >59 years old in Zou had a higher risk for BU than women, but men and women in the <59-year-old group were equally at risk (*11*). Our present study did not confirm sex differences in either those >50 or ≤50 years of age. These 2 studies differ in design: the earlier study (*11*) of the general population of Zou showed that 43% were boys and men. In this study, the percentage of male control subjects was slightly higher (47%) and may suggest that no differences in risk exist. The percentages of school-age and nonschool-age male BU patients increased relative to controls, while the percentages of female farmers and school-age and nonschool-age girls were nearly equal. Although we did not evaluate specific work or duties, girls often carry water and perform other tasks, but young boys usually play and frequent contaminated environments. Reduced risk for BU in housewives may be related to their reduced contact with contaminated environments. Differences between age and occupation may reflect differences in age-specific frequency and intensity of exposure to *M. ulcerans*.

From 1997 to 2001, CSNG received patients mainly from the Zou Region (*6*). Few patients come to CSNG from remote areas for treatment of diseases other than BU. The total number of BU patients is likely underestimated because of complex socioeconomic factors involved in care seeking and treatment of chronic diseases such as BU (*21*).

Studies that evaluated the presence or absence of BCG scars to determine vaccination status reported that scars develop in most vaccinated persons (*22*,*23*). No association was found between BU and BCG status in children <5 years old after adjustment for age. In persons ≥5 years old, a BCG scar resulted in a risk factor of 2.5 for BU compared with those without a BCG scar (Table 5). Our results

differ from those of 2 prospective studies that reported that BCG vaccination partially protects against BU (*24*,*25*). However, this protection seemingly decreased after 6 months (*24*).

In children in Benin ≥5 years old, a BCG scar may represent a risk factor for BU. Vaccination may rarely introduce *M. ulcerans* intradermally from contaminated skin (*26*). At CSNG, BU developed in several patients at the site of BCG vaccination (J. Aguiar, C. Steunou, C. Zinsou, unpub. data).

The efficacy of BCG in preventing dissemination of tuberculosis in children is well known (*27*). BCG vaccination at birth also provides protection against the development of *M. ulcerans* osteomyelitis (*28*,*29*). The disparity in the results of our case-control study may reflect known variations of efficacy of BCG (*30*). Factors related to host and the causative agent may explain varying protection of BCG against BU (*31*). An alternative explanation is variation of BCG coverage for controls (general population or hospital controls) compared with BU patients (*32*). From 1993 to 1999, BCG vaccination coverage in Zou was stable (≈90%–100%). A matched case-control study in Benin of neighborhood controls and BU cases to assess the efficacy of BCG vaccination at birth is in progress. This ongoing study will evaluate the validity of our findings (*33*).

Cases and controls are likely to have similar equal exposures to environmental transmission factors for BU. Results of our study are consistent when comparing data from other regions in Benin or only from Zou.

This case-control study confirms findings of previous studies, which indicated that children <15 years of age are at highest risk for acquiring BU (*1*,*6*), and that in areas endemic for BU, exposure to unprotected water is a risk factor for the disease. (*1*,*14*). Our study demonstrates that the association between BU and exposure to unprotected water is not as strong in patients ≥50 years of age than in other age groups. This suggests that BU in older people may be related to reactivation of latent infections by *M. ulcerans*. This study also demonstrates differences between sexes, which may be associated with age-influenced domestic, agricultural, and recreational activities. BCG vaccination is a risk factor for BU in persons ≥5 years old. Because of the diversity of conditions encountered among controls, we do not believe that these conditions have influenced our results.

Programs for provision of protected water for drinking and domestic use would have the greatest effect on control of BU in Benin. This effect could be accomplished by drilling wells and supplying pumps. Appropriate educational programs that promote behavioral changes should also reduce the frequency of BU.

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Histologic Features and Immunodetection of African Tick-bite Fever Eschar

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African tick-bite fever (ATBF) is a rickettsiosis caused by *Rickettsia africae*. We describe histologic features and immunodetection of *R. africae* in cutaneous inoculation eschars from 8 patients with ATBF, which was diagnosed by culture or association of positive PCR detection and positive serologic results. We used quantitative image analysis to compare the pattern of inflammation of these eschars with those from Mediterranean spotted fever. We evaluated the diagnostic value of immunohistochemical techniques by using a monoclonal antibody to *R. africae*. ATBF eschars were histologically characterized by inflammation of vessels composed mainly of significantly more polymorphonuclear leukocytes than are found in cases of Mediterranean spotted fever ($p<0.05$). Small amounts *R. africae* antigens were demonstrated by immunohistochemical examination in 6 of 8 patients with ATBF. Neutrophils in ATBF are a notable component of the host reaction, perhaps because ATBF is a milder disease than the other rickettsioses. Immunohistochemical detection of rickettsial antigens may be useful in diagnosing ATBF.

African tick-bite fever (ATBF), a recently rediscovered rickettsiosis of the spotted fever group, is caused by *Rickettsia africae*, an obligate intracellular, gram-negative, rod-shaped bacterium, transmitted by cattle ticks of the *Amblyomma* genus (*1–5*). ATBF is highly prevalent in Africa and often affects visitors to this region (*1,6–8*). In patients, ATBF manifests as an acute, febrile, and influenzalike illness, frequently accompanied by severe headache, prominent neck muscle myalgia, inoculation eschars (which appear as black crusts surrounded by a red halo at the site of the tick bite), and regional lymphadenitis (*5*). As many as 50% of patients have multiple eschars (*1*).

Inoculation eschars occur at the sites of tick bites and consist of a focus of epidermal and dermal necrosis ≈1 cm in diameter; they represent the portal of entry of the infectious agent into the host and the first site of challenge between the infected human host and the bacterium. Eschars have been reported and their pathologic features have been described in other spotted fever rickettsioses such as Mediterranean spotted fever, North Asian tick typhus, or Queensland tick typhus (*9–11*). Despite several clinical descriptions of ATBF, the pathologic features of the inoculation eschar have not yet been described. To determine the pathologic characteristics associated with *R. africae* infection at the cutaneous level, we analyzed skin biopsy specimens of patients with confirmed ATBF and compared the results with those from patients with Mediterranean spotted fever, the rickettsiosis caused by *R. conorii*, with respect to the pattern of inflammatory reaction. Moreover, we performed immunohistologic testing for localization of the bacteria in skin biopsy specimens. The specific detection of *R. africae* may be useful when ATBF is suspected, especially in differentiating this rickettsiosis from other spotted fever group rickettsioses.

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Patients, Materials, and Methods

Case Definition

Patients referred to our laboratory from 1999 to 2004 were classified as definite cases of *R. africae* infection if this rickettsia was isolated from clinical specimens or a positive PCR detection was associated with a positive serologic test result. Of these, we selected those for whom inoculation eschar biopsy specimens had been formalin fixed and paraffin embedded for histopathologic analysis.

Serology, PCR, and Culture

Immunofluorescence assays, the reference diagnostic method, were carried out by using both *R. conorii* strain Seven (Malish, ATCC VR-613T) and *R. africae* strain

ESF-5 as antigens (*6*). Titers of 64 for IgG and 32 for IgM in patient serum specimens were considered evidence of recent infection by a *Rickettsia* sp. (*12–14*). Western blotting and cross-absorption procedures were performed (*15–17*). The microorganism was isolated in the manner previously reported (*6*). DNA was extracted from material frozen at –80°C. DNA was extracted from ground eschar biopsy specimens by using the QIAmp Tissue kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's recommendations. These DNA extracts were used as templates in a PCR assay incorporating the *ompA* primers 190-70 and 190-701 (*18*). For negative controls, we used sterile water processed as described and DNA extracted from a heart valve from a patient with degenerative valvulopathy. For positive controls, we used DNA from *R. montanensis* strain M/5-6. Testing was performed in a blinded manner. All positive PCR products were sequenced in both directions (*19*). When regular PCR did not detect rickettsiae in skin biopsy specimens, we used a nested PCR incorporating the *omp*A-amplifying primer sets AF1F–AF1R and then AF2F–AF2R for the nested amplification (*1*). Every 6 specimens, we incorporated the above-described negative controls. To avoid contamination, we did not include any positive control in the assay. Positive nested PCR products were identified by sequencing with the AF2F–AF2R primer pair.

Histologic Analysis and Immunohistochemical Detection of *R. africae*

Formalin-fixed, paraffin-embedded skin biopsy specimens of the inoculation eschars were cut to 3 μm in thickness and stained with hematoxylin-eosin-saffron by routine staining methods. Serial sections of each tissue specimen were also obtained for special staining or immunohistochemical investigations. Immunohistochemical analysis was performed on paraffin-embedded skin biopsy sections by use of the monoclonal mouse antibody AF8-F3 produced against *R. africae* (*20*). The specificity of this antibody has been tested on a variety of rickettsiae, and cross-reactivity was found only with *R. parkeri* and *R. sibirica* (*20*). The immunohistologic procedure, in which an immunoperoxidase kit was used, has been described elsewhere (*21*). Briefly, after deparaffinization, each tissue section was incubated with the monoclonal anti–*R. africae* antibody diluted 1:1000 in phosphate-buffered saline. After the sections were incubated with the primary antibody, immunodetection was performed with biotinylated immunoglobulins, followed by peroxidase-labeled streptavidin (HistoStain plus kit, Zymed, Montrouge, France) with amino-ethyl-carbazole as substrate. The slides were counterstained with Mayer hematoxylin for 10 min. For each case, 3 level tissue sections were systematically evaluated by immunohistochemical analysis, and a negative control was created by using an irrelevant monoclonal mouse antibody. Moreover, to test the specificity of our monoclonal antibody, skin biopsy specimens from patients with Mediterranean spotted fever (15 cases), acute eczematous dermatitis (2 cases), psoriasis (2 cases), and lichen planus (1 case) served as negative controls.

Quantitative Image and Statistical Analyses

To characterize the immune response in inoculation eschars during ATBF and Mediterranean spotted fever, paraffin sections were stained with the polymorphonuclear leukocyte marker CD15 (Immunotech, Marseille, France), the macrophage marker CD68 (Dako, Trappes, France), the T-lymphocyte marker CD3 (Dako), the B-lymphocyte marker CD20 (L26, Dako), and the endothelial cell marker Factor VIII–related antigen (Dako) by using the peroxidase-based method described above. The antibodies anti-CD15 and anti-CD3 were ready to use, whereas the antibodies anti-CD68, anti-CD20, and anti-Factor VIII–related antigen were used at a working dilution of 1:1000.

The evaluation of the proportion of polymorphonuclear leukocytes, macrophages, T lymphocytes, and B lymphocytes, as well the relative proportion of neovascularization in skin tissue specimens, was determined by quantitative image analysis (*22*). In brief, histologic images were digitized and transferred to a computer system. Using the image analyzer SAMBA 2005 (SAMBA Technologies), which is a specific interactive program that provides visual control of analysis, we analyzed the CD15-positive, CD68-positive, CD3-positive, CD20-positive, and Factor VIII–positive surfaces in tissue sections to determine the percentages of the total surface area covered by neutrophils, macrophages, T and B lymphocytes, and endothelial cells, respectively. For each set of observations, the surfaces of 10 randomly chosen areas were studied at a magnification ×100, and the surface areas of each immunohistologic parameter were measured. The average areas were calculated by comparison with the area of the whole tissue sample. The Mann-Whitney U test was used for the statistical comparisons of values that were obtained for each immunohistologic parameter for skin biopsy specimens from patients with ATBF and patients with Mediterranean spotted fever; $p<0.05$ was considered significant.

Value of Immunohistochemical Techniques

The sensitivity, specificity, and positive and negative predictive values of immunohistochemical techniques for the diagnosis of ATBF were calculated. The sensitivity of immunohistochemical analysis was compared to that of culture, regular PCR, nested PCR, and serologic testing by using the Fisher exact test.

Results

Skin biopsy specimens from 8 patients with ATBF, from 1999 to 2004, obtained from black crusts corresponding to inoculation eschars, were positive for *R. africae* by culture (4 patients) or PCR and serologic tests (4 patients). The age of the eschars at the time of biopsy varied from 5 to 10 days. The epidemiologic and clinical features of the cases in the 8 patients with ATBF are summarized in Table 1. The mean age of patients was 46.87 years (standard deviation 13.17 years, range 27–63 years); 4 were men and 4 were women. A history of tick bite was reported by 3 (37.5%) of 8 patients. Clinical features included fever in 6 (75%) of 8 patients, a vesicular cutaneous rash in 5 (62.5%) of 8 patients, regional lymphadenopathies in 3 (42.85%) of 7 patients, and headache and myalgia in 5 (71.42%) of 7 patients. The inoculation eschar was single in 3 (37.5%) of 8 patients and multiple in 5 (62.5%) of 8 patients. Erythema and swelling surrounded the eschar in 7 (87.5%) of 8 patients. Details of the results of laboratory tests, culture, serologic tests, PCR, and immunohistochemical tests are found in Table 2.

Histologic and Immunohistochemical Analyses

Eschars of ATBF cases were histologically dominated by vascular injury and perivasculitis, which were detected in all skin biopsy specimens examined. The host response to the vascular injury was manifested as intramural and perivascular infiltration by polymorphonuclear leukocytes, small lymphocytes, and macrophages (Figure 1). Vascular damages were endothelial swelling and vascular fibrinoid necrosis (Figure 2). Mural and occlusive fibrin thrombi were observed in a few blood vessels. Vascular injuries were associated with cutaneous necrosis (Figure 2). Moderate-to-severe cutaneous necrosis was present in 7 of 8 cutaneous biopsy specimens. When cutaneous necrosis was found, it was associated with vascular thrombosis. No hemorrhages were noted. Quantitative immunohistochemical analysis showed that the immune response in skin biopsy specimens from patients with ATBF more predominantly involved polymorphonuclear leukocytes than did specimens from patients with Mediterranean spotted fever ($p<0.01$, Figures 3 and 4). In contrast, the inflammatory infiltrates in patients with Mediterranean spotted fever were mainly characterized by macrophages ($p = 0.04$, Figure 4) and T and B lymphocytes. The relative amounts of vessel formation were similar in the 2 diseases.

Six of 8 eschar biopsy specimens contained *R. africae* detected by immunohistochemical analysis (Table 2). Rickettsial antigen was observed in the endothelium and inflammatory cells organized in and around blood vessels (Figure 5). None of the control skin biopsy samples, including the *R. conorii*–infected cutaneous specimens, showed immunoreactivity with the monoclonal mouse antibody AF8-F3. Finally, no statistical correlations were found between the age of the cutaneous lesions and the amount and type of inflammatory cell infiltrate as well as the amount of rickettsial antigen found in the lesion.

The sensitivity, specificity, and positive and negative predictive values of immunohistochemistry were 75%, 100%, 100%, and 91%, respectively. The sensitivity of immunohistochemical tests was not significantly different from that of culture (6/8 vs 4/8, $p = 0.6$), regular PCR (6/8 vs 6/8, $p = 1$), nested PCR (6/8 vs 8/8, $p = 0.5$), and serology (6/8 vs 4/8, $p = 0.6$) (*1*,*12*,*28*).

Discussion

In some rickettsioses, the inoculation eschar is the site of an intense rickettsial multiplication and thus is the preferred biopsy specimen for studying the pathologic features of *Rickettsia*-induced infection and for detecting the bacteria by immunohistologic tests as well as for carrying out isolation procedures or genomic detection. As demonstrated before for many rickettsioses, the cutaneous damages at the inoculation site were histologically dominated by vasculitis and necrotic features (*11*). Rickettsial invasion of endothelial cells probably represents the first step of infection (*7*). Subsequent damage to the endothelium is followed by endothelial-cell activation and perivascular infiltration of lymphocytes, macrophages, and polymorphonuclear leukocytes, resulting in inflammatory vasculitis of dermal vessels, the histopathologic hallmark of rickettsial disease, and possibly thrombosis (*23*). However, in contrast with the other rickettsial diseases that are characterized by perivascular infiltration of T cells and

Table 1. Epidemiologic and clinical features of cases in 8 patients with African tick-bite fever

Patient no.	Age (y), sex	Country of exposure	No. eschars, site	Fever	Cutaneous rash	Regional lymphadenopathy	Headaches and myalgia
1	55, M	South Africa	>1, unknown	+	–	–	–
2	47, F	South Africa	1, leg	+	Vesicular	–	–
3	63, M	Swaziland	2, knee and shoulder	–	Vesicular	+	+
4	56, F	Swaziland	>1, unknown	+	Vesicular	+	+
5	28, F	South Africa	2, leg and chest	+	–	–	+
6	27, M	South Africa	1, leg	–	Vesicular	+	+
7	45, F	South Africa	1, calf	+	Vesicular	Unknown	Unknown
8	54, F	South Africa	3, groin and leg	+	–	–	+

Table 2. Biologic and pathologic features of cases in 8 patients with African tick-bite fever*

Patient no.	Culture	Serology (IFA or Western blot)	Regular PCR	Nested PCR	IHC tests
1	–	+	+	ND	+
2	+	–	+	ND	+
3	+	–	+	ND	+
4	–	–	+	ND	+
5	–	+	–	+	–
6	–	+	–	+	–
7	–	+	+	ND	+
8	+	–	+	ND	+

*IFA, immunofluorescence assay; IHC, immunohistochemical; ND, not done.

macrophages, with some B lymphocytes and few neutrophils (*10*,*24*–*26*), the cutaneous damage of ATBF show vasculitis with polymorphonuclear leukocyte–rich inflammation. The predominance of neutrophils in inflammatory infiltrates may explain the importance of the local inflammation clinically observed at the site of inoculation, accompanied by the regional lymphadenitis.

By using immunohistochemical techniques, we demonstrated *R. africae* in cutaneous biopsy specimens from patients with ATBF. In accord with its obligate intracellular location, no extracellular organisms were observed in cutaneous biopsy specimens. Few bacterial antigens were found in vascular and perivascular locations within the cytoplasm of endothelial and inflammatory cells. In spite of the small amount of antigens detected, the inflammatory and necrotic cutaneous damage was histologically extensive. Our data indicate that *R. africae* replicates poorly in human tissues, likely because of its mild pathogenicity and the strong innate immune response. The local destruction of bacteria by the inflammatory reaction may explain the benign outcome of the disease.

In our study, immunohistochemical techniques had a sensitivity of 75%, a specificity of 100%, and a positive predictive value of 100%. Serologic tests, the most widely used diagnostic method for rickettsioses, had a sensitivity of 56% in early samples (*12*). Although our data suggested that immunohistochemical analysis might be more sensitive than serology in early samples, statistical analysis showed no significant difference between the 2 techniques. Regarding diagnostic methods applicable to skin biopsy specimens, immunochemical techniques were also more sensitive than culture (41%), which is restricted to specialized laboratories equipped with biohazard facilities; such techniques were also more sensitive than regular PCR (47%) (*27*). Moreover, the techniques exhibited a sensitivity similar to that of nested PCR (73.5%), previously found to be the most efficient diagnostic technique for spotted fever rickettsioses (*27*).

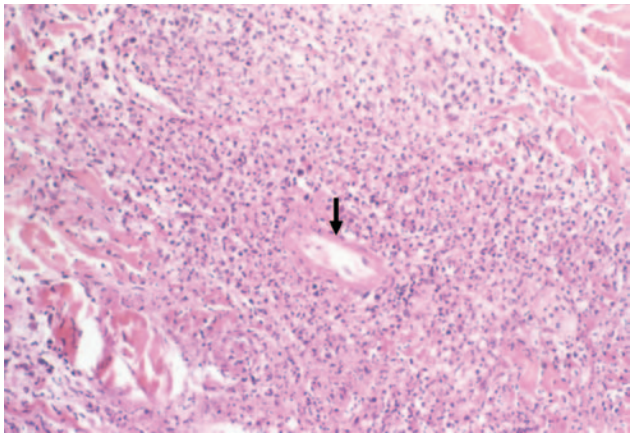

Figure 1. Fibrinoid necrosis of a vessel in the dermis (arrow) with perivascular inflammatory infiltrates mainly composed of polymorphonuclear leukocytes (hematoxylin-eosin-saffron; original magnification ×250).

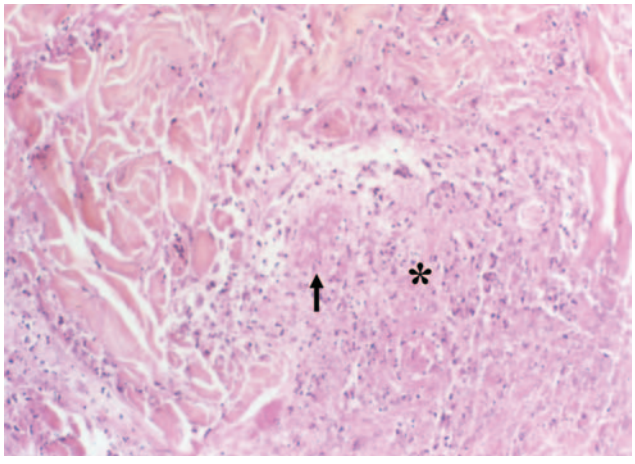

Figure 2. Coagulative necrosis (*) of the dermis surrounding necrotic vessels (arrow) (hematoxylin-eosin-saffron; original magnification ×250).

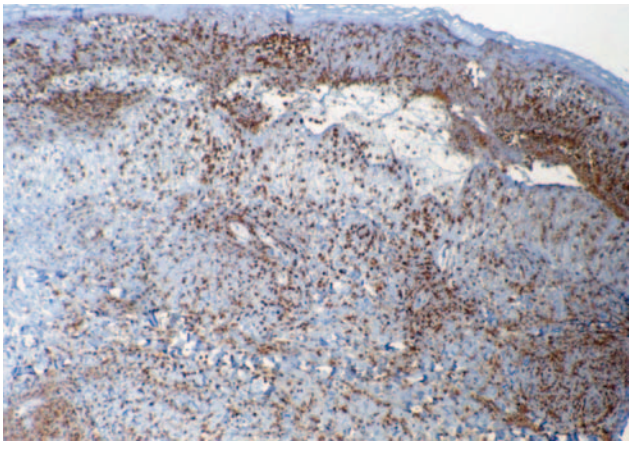

Figure 3. Inoculation eschar from a patient with African tick-bite fever showing numerous dermal inflammatory infiltrates mainly composed of polymorphonuclear leukocytes (immunoperoxidase staining with an anti-CD15 antibody; original magnification ×100).

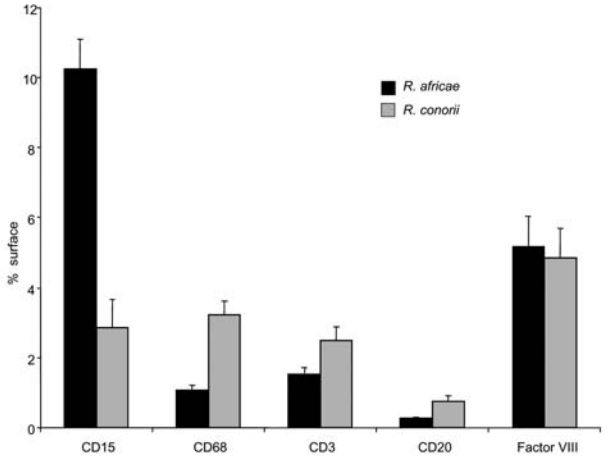

Figure 4. Quantification of inflammatory changes in inoculation eschars from patients with African tick-bite fever (*Rickettsia africae*, n = 8) and patients with Mediterranean spotted fever (*R. conorii*, n = 15). Surface areas expressing CD15, CD68, CD3, CD20, and Factor VIII were quantified after immunostaining. Quantification of each parameter was evaluated by computer-assisted analysis of digitized microscopic images. Results were normalized and expressed as a percentage of the total skin tissue surface area. Columns represent mean values ± standard error.

As Mediterranean spotted fever caused by *R. conorii* is endemic in the same regions of Africa as tick-bite fever, differentiation of the 2 syndromes by characterization of their etiologic agents may be useful for diagnostic and epidemiologic studies (*4*,*28*,*29*). The polymorphonuclear leukocyte–rich vasculitis, which dominates the histologic features of the inoculation eschar during ATBF, could suggest the diagnosis of this rickettsiosis but is not specific.

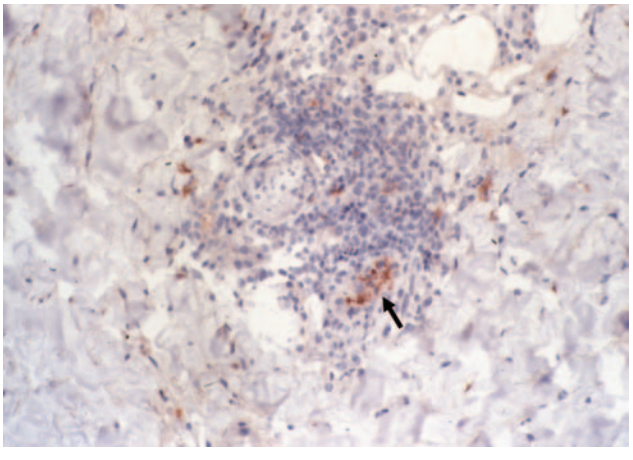

Figure 5. Immunohistochemical detection of *Rickettsia africae* in the inoculation eschar of a patient with African tick–bite fever. Note the location of the bacteria in the endothelial and inflammatory cells of a blood vessel in the dermis (arrow) (monoclonal rabbit anti-*R. africae* antibody used at a dilution of 1:1,000 and hematoxylin counterstain; original magnification ×250).

The usual method for the diagnosis of rickettsioses is serologic testing. However, serologic cross-reactions are common among the rickettsiae in the spotted fever group, particularly between *R. africae* and *R. conorii* infections (*6*). Monoclonal antibodies had been developed to *R. africae* for use in assays to distinguish between *R. conorii* and *R. africae* in culture and skin biopsy samples (*20*). In this study, we used a monoclonal antibody produced in our laboratory to distinguish ATBF from Mediterranean spotted fever at the histologic level by immunohistologic methods. We presented the pathologic description of the first series of inoculation eschars from skin biopsy specimens of patients with ATBF. We showed that cutaneous damage is dominated by vasculitis, thrombosis, cutaneous necrosis, and a polymorphonuclear leukocyte–rich inflammatory reaction. Immunohistochemical detection of rickettsial antigens may be useful in diagnosing ATBF. Pathologists should now consider ATBF, a recently rediscovered rickettsiosis, during histologic analysis of inoculation eschars, especially in patients with a recent stay in sub-Saharan Africa.

Dr Lepidi is an associate professor of pathology at the Université de la Méditerranée, Marseille. His research interests include the pathology of infectious diseases, especially bacteria with intracellular development and rickettsioses.

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Lymph Node Biopsy Specimens and Diagnosis of Cat-scratch Disease

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We report microbiologic analysis of 786 lymph node biopsy specimens from patients with suspected cat-scratch disease (CSD). The specimens were examined by standard, cell culture, and molecular methods. Infectious agents were found in samples from 391 (49.7%) of 786 patients. The most commonly identified infectious agent was *Bartonella henselae* (245 patients, 31.2%), the agent of CSD. Mycobacteriosis was diagnosed in 54 patients (6.9%) by culture and retrospectively confirmed by using a specific real-time PCR assay. Neoplasm was diagnosed in 181 specimens suitable for histologic analysis (26.0%) from 47 patients. Moreover, 13 patients with confirmed *Bartonella* infections had concurrent mycobacteriosis (10 cases) or neoplasm (3 cases). A diagnosis of CSD does not eliminate a diagnosis of mycobacteriosis or neoplasm. Histologic analysis of lymph node biopsy specimens should be routinely performed because some patients might have a concurrent malignant disease or mycobacteriosis.

Lymph node enlargement is a common medical problem. Infections caused by bacterial, viral, and protozoal agents are the most typical cause of localized lymphadenopathy, but malignancies or lymphoproliferative diseases are also often found (*1*). Physicians must differentiate malignant lymphadenopathies or infectious diseases that require special care from benign reactive lymphadenopathy or self-limiting adenitis.

In a large number of patients, the causes of lymphadenopathy remain undiagnosed. Causes of lymphadenopathy other than neoplasm that require urgent medical attention include tuberculosis and HIV infection. During the past 15 years, *Bartonella henselae*, the causative agent of cat-scratch disease (CSD), has been reported as a common cause of localized lymphadenopathy (*1–3*). Diagnostic techniques for *Bartonella*-related infections include culture of the pathogen (*4*,*5*), detection of organisms in lymph nodes by immunofluorescence (*6*), molecular techniques including PCR amplification of *Bartonella* spp. genes (*7*,*8*), and serologic analysis (*9*,*10*). *B. henselae* is not commonly isolated from CSD patients (*4*,*11*), and PCR-based detection of various target genes of *Bartonella* species in tissue specimens has become the most widely accepted way of diagnosing CSD (*7*,*8*).

Serologic analysis is a minimally invasive diagnostic technique that has been extensively evaluated for the diagnosis of CSD (*9*,*10*,*12*). The sensitivity of serologic tests varies from 1 laboratory to another, ranging from nearly 100% to <30% (*9*). Specificity may also vary, and a specificity ≥95% may be achieved by using commercial tests with immunoglobulin G cutoff titers ≥128 (*10*).

As a national reference center for rickettsioses and bartonelloses, we routinely receive lymph node biopsy specimens from patients with suspected CSD. In this study, we analyzed a large collection of lymph node biopsy samples obtained from January 2001 through August 2005 using microbial cultures (blood agar culture and cell culture) and 16S rDNA- and *Bartonella*-specific PCR. Our objective was to define the frequency of *B. henselae* and other bacterial infections in patients with suspected CSD in France.

Methods

Patients

We studied lymph node biopsy specimens from patients with suspected CSD that were collected from January 2001 through August 2005. Tissues specimens sent to our

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reference center were obtained from both hospitalized patients and outpatients throughout France. We receive either the entire lymph node or a fragment of it; the specimens were sent either frozen or in transport media. This factor is crucial because most of the specimens received were not in suitable condition for histologic analysis. A definitive diagnosis of CSD was defined as a biopsy sample that was positive by PCR for 2 different target genes of *Bartonella* spp. (*6*). If a specimen had been previously analyzed and *B. henselae* was reported (*7*), the specimen was excluded from the present study.

Detection of *Bartonella* DNA in Tissue Specimens

Total genomic DNA was extracted from samples with a QIAamp tissue kit (Qiagen, Hilden, Germany) as previously described (*7*). Samples were handled under sterile conditions to avoid cross-contamination. Genomic DNA was stored at 4°C until used as template in PCR assays. The primers used for *B. henselae* amplification and sequencing (internal transcribed spacer [ITS] region and *pap31* gene) have been previously evaluated (*6*,*7*). Up to 10 samples were tested, along with negative controls (DNA from noninfected lymph nodes and sterile water) and a positive control (DNA from *B. elizabethae* for the ITS region, GenBank accession no. L35103, and DNA from *B. henselae* Houston-I for the *pap31* gene, GenBank accession no. AF001274).

Detection of Bacteria in Tissue Specimens

Nucleic acids were extracted with a QIAamp tissue kit (Qiagen) and PCR performed with universal 16S rDNA primers fD1 and rp2 (Eurogentec, Seraing, Belgium) (*13*) and Taq DNA polymerase (GIBCO-BRL Life Technologies, Gaithersburg, MD, USA). Amplification and sequencing of products were conducted as previously described (*14*). Up to 10 samples were tested, along with negative controls (noninfected lymph node and sterile water) and positive controls (*B. henselae* Houston-I and *Staphylococcus aureus* (ATCC 29213). The 16S rDNA sequences obtained were compared with all bacterial 16S rRNA sequences available in the GenBank database by using the Blastn version 2.2.2 program (National Center for Biotechnology Information, Bethesda, MD, USA). The efficiency of DNA extraction and presence of inhibitors in samples that were negative by PCR were tested by using primers that targeted a fragment of the human β-globin gene as previously described (*15*).

Detection of *B. henselae* in Lymph Nodes

We confirmed *B. henselae* in lymph nodes of patients with CSD by using a specific monoclonal antibody for *B. henselae* as previously described (*6*). The slides were air-dried and fixed with methanol for 10 minutes at room temperature before testing with an immunofluorescence assay (*6*). The sensitivity and specificity of this assay and antibody were previously reported to be 79.6% and 92.5%, respectively (*6*).

Culture Methods

Lymph node biopsy specimens were placed on blood agar plates, incubated at 37°C in an atmosphere of 5% CO_2, and examined weekly for growth during a 2-month period. This process resulted in isolation of either *Bartonella* or mycobacteria (*16*). Specimens were also placed on human embryonic lung cells in shell vials and incubated at 37°C in an atmosphere of 5% CO_2 as previously described (*4*,*17*). From January 2002 to August 2005, specimens were also incubated onto horse blood agar supplemented with hemin (100 mg/L). This procedure has been reported to improve the isolation rate of *B. henselae* and can also support growth of rapidly growing mycobacteria (*11*,*16*). Specimens were also cultured under anaerobic conditions. *Bartonella* isolates were identified by PCR and sequencing as described above; other bacterial isolates were identified by using standard bacteriologic methods. Samples from which mycobacteria were isolated were reanalyzed retrospectively by real-time PCR with modified primers and probes targeting the ITS region as previously described (*18*).

Histologic Analysis

Samples that had not been frozen (181 specimens) were fixed in formalin and processed for histologic analysis. Stains used included Gram, hematoxylin and eosin, periodic acid–Schiff, Ziehl-Neelsen, and Warthin-Starry.

Statistical Analysis

Two groups of patients were defined for demographic data comparisons: CSD patients (detection of *Bartonella* DNA) and non-CSD patients (no detection of *Bartonella* DNA). For data comparison, the Student *t* test or χ^2 test was performed by using EpiInfo version 6.0 software (Centers for Disease Control and Prevention, Atlanta, GA, USA).

Results

Diagnoses in Patients with Lymphadenopathy

We tested 786 lymph node biopsy specimens from patients with suspected CSD. Only 181 specimens were suitable for histologic analysis. Neoplasm was diagnosed by histologic analysis in 47 (26.0%) of 181 patients (6 with skin carcinomas, 1 with acute leukemia, 24 with lymphomas, 12 with Hodgkin disease, and 4 with Kaposi sarcoma). Bacteria were cultured from 143 specimens (18.2%), and mycobacteria were the most frequently recovered organisms (54 [6.9%] of 786) on blood agar or

by shell vial culture (Table 1). The 54 nodes that contained mycobacteria were retrospectively confirmed by using real-time PCR targeting the ITS region. Other common bacteria recovered either by culture or PCR were staphylococci (26 cases) and *Propionibacterium acnes* (15 cases). *B. henselae* was cultured and successfully passaged from 1 lymph node, and *B. quintana* was cultured and amplified from 1 lymph node. Fastidious bacteria were cultured from lymph nodes by the shell vial cell culture: 2 isolates of *Coxiella burnetii* and 1 isolate of *Francisella tularensis*, which has been previously reported (*19*) (Table 1). Anaerobic bacteria cultured from lymph nodes included *Fusobacterium* spp. (4 specimens), *Prevotella* sp. (1 specimen), and *Clostridium perfringens* (1 specimen).

Amplification of the 16S rDNA gene for common bacteria was performed on all specimens. Positive results were obtained for 236 patients (30.0%), and *B. henselae* was the most frequently amplified bacterium (122 cases, 51.7%). Other bacteria commonly detected included mycobacteria, staphylococci, streptococci, and *P. acnes* (Table 1). Fastidious bacteria were isolated from 5 lymph nodes: *C. burnetii* (3 cases), *F. tularensis* (1 case), and *Tropheryma whipplei* (1 case). These 5 diagnoses were confirmed by a second specific PCR with primers and probes routinely used in our laboratory. Using specific primers for the ITS region and *pap31* gene of *Bartonella* spp., we identified *Bartonella* spp. in 245 patients (31.2%), including 122 patients identified by PCR with primers for the 16S rDNA gene. No discordance was observed between the ITS region and the *pap31* gene.

When compared with specific detection of *Bartonella* DNA, specificity of the 16S rDNA PCR was 100% but sensitivity was low (49.8%, 122 of 245 lymph nodes were positive). Positive and negative controls showed expected results in all tests. All but 1 of the sequences of the ITS region and *pap31* genes we obtained were identical to those of *B. henselae* reported in GenBank. In 1 patient, the sequences obtained were identical to those of *B. quintana*. Among these 245 samples positive for *Bartonella*, 216 were also tested by direct immunofluorescence assay with monoclonal antibodies to *B. henselae*, of which 166 (76.9%) were positive.

A total of 391 (49.7%) of 786 patients had an infectious disease (including the 10 patients whose specimens were *B. henselae*–positive by PCR and showed mycobacterial infection), 47 had neoplasm (including 3 specimens with *B. henselae*–positive PCR result), and 351 (44.6%) had no identified cause for their lymphadenopathy (Table 1). On the basis of these results, we divided the patients into 2 groups: patients with a positive PCR result for *Bartonella* (n = 245) (CSD group) and the remaining patients (n = 541) (non-CSD group).

Table 1. Results of culture and PCR assays of 786 biopsy lymph node specimens*

Diagnosis or infection	Positive culture	*Bartonella*-positive PCR	16S rDNA–positive PCR	Total
CSD	1	244	122	244
Bartonella quintana	1	1	1	1
Q fever	2	0	3	3
Tularemia	1	0	1	1
Abiotrophia adjacens	2	0	2	2
Actinomyces	1	0	1	1
Pasteurella multocida	2	0	2	2
Mycobacterial infection	54	0	32	54
Staphylococcus aureus	16	0	16	16
Coagulase-negative *Staphylococcus*	15	0	10	23
Streptococcus pyogenes	10	0	10	10
Fusobacterium spp.	4	0	4	4
Nocardia asteroïdes	1	0	1	1
Propionibacterium acnes	15	0	7	16
Prevotella sp.	1	0	1	1
Clostridium perfringens	1	0	1	1
Tropheryma whipplei	0	0	1	1
Miscellaneous	21	0	21	21
Neoplasm	0	0	0	47
Unknown	0	0	0	350
Total	148	245	236	449

*CSD, cat-scratch disease. Among 244 specimens PCR-positive for *B. henselae*, 10 showed a concurrent mycobacterial infection and 3 showed a neoplasm.

Comparison of Demographic Data between Patient Groups

The mean ± standard deviation (SD) age was 30.2 ± 20.4 years (range 1–94 years) in 245 patients with proven *B. henselae* or *B. quintana* lymphadenopathy (CSD group) versus 31.6 ± 20.7 years (range 4 months to 86 years) in the non-CSD group. Most patients with *B. henselae* CSD were <25 years of age ($p = 0.032$) (Figure 1). The mean ± SD ages of patients with neoplasm (46.2 ± 22.6 years, range 7–86 years) and mycobacterioses (39.5 ± 22.2 years, range 1–84 years) were greater than the mean ± SD age of patients with CSD ($p<0.05$ by Student *t* test) (Table 2). The sex ratio (male:female) was 1.28 in the CSD group and 1.50 in the non-CSD group, but this difference was not significant ($p>0.05$) (Table 2). In the CSD group, 89 of the lymph node biopsy specimens were from axillary nodes

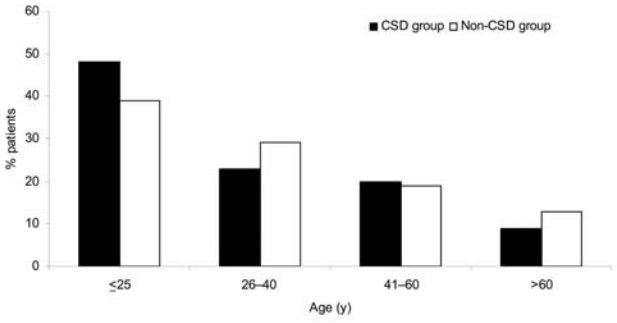

Figure 1. Distribution of patients by age and group. Cat-scratch disease (CSD) group, patients with *Bartonella*-positive PCR results in lymph node samples; Non-CSD group, patients with *Bartonella*-negative PCR results. For patients ≤25 years of age, p = 0.032 for CSD group versus non-CSD group.

(36.3%), 75 were from inguinal nodes (30.6%), and 81 were from cervical nodes (33.1%).

We found that 13 of 245 patients with CSD had concurrent lymph node disease (Table 2). Ten had mycobacteriosis proven by culture (5 with *M. tuberculosis*, 3 with *M. avium*, 1 with *M. fuerthenensis*, and 1 with *M. gordonae*), and 3 had neoplasm (2 with lymphoma and 1 with Hodgkin disease). The mean ± SD age of these 13 patients (49.7 ± 16.0 years, range 27–72 years) was higher than the mean ± SD age of the remaining 232 patients with only CSD ($p<0.05$ by Student *t* test). Only 4 lymph node biopsy specimens from the 10 patients with concurrent mycobacteriosis were positive by Ziehl-Neelsen staining. Six of 10 lymph node biopsy specimens were positive in a direct immunofluorescence assay with monoclonal antibodies for *B. henselae* (Figure 2) as previously described (*6*).

Of the 3 patients with CSD and concurrent neoplasm, a positive PCR result for the 16S rDNA gene was obtained with DNA from 1 lymph node (*B. henselae*). Two of 3 lymph nodes were positive in a direct immunofluorescence assay with monoclonal antibodies to *B. henselae* as described previously (*6*). As expected, the number of patients with either mycobacteriosis or neoplasm in the non-CSD group was higher than in the CSD group (p = 0.014; n = 181 patients).

Discussion

Culture and PCR were used to examine lymph node biopsy specimens from patients with suspected CSD. These methods, i.e., blood agar and cell culture (*20*), molecular biology with PCR for the 16S rDNA gene (*14*), PCR with 2 specific genes from *Bartonella* (*6*,*7*), and histologic analysis (*20*), have been previously validated and are routinely used for examination of lymph node samples. Our report describes an extensive study on lymph nodes using culture, 16S rDNA PCR amplification, and amplification of target genes of *Bartonella* spp.

Our objective was to define all bacterial causes of lymphadenopathies for samples initially sent to our center for detection of CSD. In the patients we studied, 50% had infectious diseases, and the most common causative agent was *B. henselae*; ≈30% of suspected patients were PCR positive (CSD group). Sensitivity of PCR with the 16S RNA gene to diagnose CSD was lower than was *Bartonella*-specific PCRs. The sensitivity of PCR assays with the 16S rRNA gene for the diagnosis of CSD has been reported to vary from 43% to 100%, depending on the primers used and the definition of a positive case (*21*,*22*). In our laboratory, PCR with specific primers against *Bartonella* genes is more sensitive and specific in the diagnosis of CSD.

In a recent study in Germany, *B. henselae* was the causative agent of head and neck lymphadenopathy in 61 (13.4%) of 454 patients (*1*). As in our study, *B. henselae* was the most common organism responsible for lymphadenopathy in adults and children (*1*). However, our higher percentage of positive PCR results was because specimens sent to our reference laboratory were from patients with suspected CSD. Many cases of CSD remain unrecognized because serologic or molecular analyses are not routinely used. We observed a low isolation rate for *B. henselae* on axenic media or in cell culture, only 1 successfully passaged isolate among the 245 PCR-positive

Table 2. Comparison of demographic data between CSD patients and non-CSD patients*

Factor	No. patients	Age, y (mean ± SD)	Sex ratio (M/F)	p value†
CSD group (total)	245	30.2 ± 20.4	1.28	
CSD alone	231	29.4 ± 19.6	1.26	
CSD plus mycobacteria	10	43.3 ± 8.2	1.0	
CSD plus neoplasm	3	57.3 ± 6.0	3.0	
Bartonella Quintana alone	1	31.6 ± 20.7		
Non-CSD group	541	39.5 ± 22.2	1.50	>0.05
Mycobacteria	44	46.2 ± 22.6	1.72	<0.05
Neoplasm	44	30.2 ± 20.4	1.30	<0.05

*CSD, cat-scratch disease; SD, standard deviation.
†Comparison of mean age of CSD group and corresponding non-CSD group.

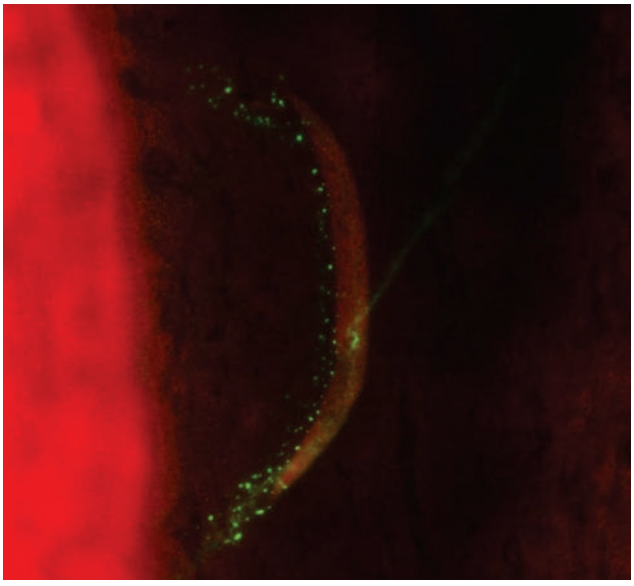

Figure 2. Detection of *Bartonella henselae* in the lymph node of a patient with cat-scratch disease and tuberculosis by direct immunofluorescent assay with a monoclonal antibody (magnification ×400).

samples, which is consistent with previous findings (*4*,*8*). This rate did not improve when we used an enriched medium designed to improve isolation of *B. henselae* (*11*). A recently developed enriched liquid medium for growth of *Bartonella* strains (*23*) may be useful in obtaining more isolates of *B. henselae* from patients with CSD. However, in many lymph nodes negative by culture, we observed bacteria by direct immunofluorescence, which suggests that bacteria in lymph nodes are not viable (*6*). Consistent with this finding was that most nodes were necrotic at histopathologic examination (data not shown). One lymph node was positive for *B. quintana* by culture and PCR as previously reported (*24*).

The long incubation time needed for isolation of *Bartonella* allows us to isolate mycobacterial strains by using blood agar culture (*16*). We found mycobacteria incidentally and not because of a specific search. Moreover, even if mycobacteria grew well in blood agar plates (*16*), sensitivity of culture from lymph nodes is not 100%. This fact means that the percentage of mycobacterial infections in our study was probably underestimated because specific PCR for mycobacteria was only performed retrospectively in culture-positive specimens. On the basis of these results, we now routinely perform Ziehl-Neelsen staining and PCR to detect mycobacteria in all specimens.

Before the discovery of *B. henselae* and the use of PCR for its diagnosis, mycobacteria were the most frequent infectious agents causing lymphadenopathy (*25*), and staphylococci and group A streptococci were the main causes of acute adenitis. In our study, mycobacteria were the second most common infectious cause of lymph node enlargement; ≥6.9% of patients were infected. The 16S rRNA PCR in our study had a lower sensitivity than culture in the diagnosis of mycobacterial infection. This finding may have resulted from sample pretreatment to adequately purify DNA (*26*). Freidig et al. found that 24 (5.7%) of 419 lymph nodes were enlarged because of mycobacterial infection (Table 3) (*27*). Similar incidences have been reported by Doberneck (*28*) and Anthony and Knowles (*29*) (Table 3). Higher incidences of mycobacterial infections (27 [16.6%] of 163 lymph node biopsy specimens) were reported by Roberts and Linsey (*25*). In our study, 76% of mycobacterial infections were *M. tuberculosis*; 54% were *M. tuberculosis* in the study by Freidig et al. (*27*). This finding is consistent with the fact that the incidence of typical and atypical mycobacterial adenitis is age dependent; typical adenitis is more common in adults, and atypical adenitis is more common in children (*30*).

Table 3. Relevant studies of causes of lymphadenopathy, 1983–2004*

Variable	Doberneck (*28*)	Roberts (*25*)	Anthony (*29*)	Freidig (*27*)	Ridder (*1*)	Chau (*36*)	This study
Years	1972–1982	1978–1983	1983	1978–1986	1997–2001	1996–2001	2001–2005
No. patients	169	163	228	419	454	423	786
Mean age, y (range)	34.6 (1–78}	(1–90)	(0–≥60)	46.7 (2–89)	34.9 (2–90)	40 (14–90)	32.0 (1–94)
Infectious diseases (%)	8/79 (10.1)	76	11 (4.8)	66 (15.8)	156 (34.4)	75 (17.7)	391 (49.7)
CSD (%)	0	0	3	0	61 (13.4)	3	245 (31.2)
Mycobacteria (%)	5/79 (6.3)	27 (16.6)	6 (2.6)	24 (5.7)	5 (1.1)	12 (2.8)	54 (6.9)
Staphylococci or streptococci (%)	3/79	41	NA	2	13	2	49
Malignant process (%)	119 (70.4)	51 (31.2%)	60 (26.3)	113 (27.0)	52 (11.5)	95 (17.3)	47 (26%)†
Undiagnosed (%)	42 (24.9)	28 (17.2)	68 (29.8)	113 (27.0)	171 (37.7)	168 (39.7)	350 (44.6)

*CSD, cat-scratch disease.
†Only 181 samples could be tested by histopathologic analysis.

Other agents found in our study were staphylococci and miscellaneous aerobic and anaerobic bacteria. Isolates of coagulase-negative staphylococci or *P. acnes* may be considered contaminants, but the remaining organisms are pathogens and should be considered causative agents of lymph node enlargement (*31*). We found that rare or fastidious organisms may be the cause of infectious adenitis. Such situations have been previously reported, especially infections with *Nocardia* spp. (*32*), *C. burnetii* (*33*), *F. tularensis* (*34*), or *T. whipplei* (*35*). Only because we used cell cultures in shell vials were we able to culture *C. burnetii* and *F. tularensis* in our study. Similarly, additional cases with these fastidious organisms, as well as 1 case of infection with *T. whipplei*, were diagnosed because of systematic use of broad-range PCR on lymph nodes.

The cause of 351 cases of lymphadenopathy in this study could not be determined. Several reasons and limitations may explain this result. First, histologic data were obtained for only 23% of the lymph node specimens because most were sent to our center frozen or were too small. In 181 specimens, neoplasms may represent >25% of cases of suspected CSD. Thus, a similar proportion of neoplasms may be present in the remaining 605 specimens. For practical purposes, neoplasm can only be diagnosed by histopathologic analysis. Thus, lymph node excision is crucial in the diagnosis of malignant processes. Another limitation of our study was that we did not test for fungi or viruses that may also represent causes of lymphadenopathies. Mycobacterial infections in our study were diagnosed by culture and confirmed retrospectively by using a real-time quantitative PCR. We believe that the systematic use of real-time PCR for detection of mycobacteria will likely increase the percentage of such infections as causes of lymphadenopathies.

Previous studies reported that the percentage of undiagnosed cases varied from 17.2% to 39.7% (Table 3), and malignant processes were more common than infectious diseases. In more recent studies, percentages of lymph node specimens with malignant processes were lower (11.5%–17.3%), and infectious diseases were more common (17.7%–48.6%) (*1*,*8*,*36*) (Table 3).

We have showed that neoplasm could be clinically misdiagnosed as CSD. This finding was probably underestimated because we had previously analyzed lymph nodes only by culture and detection of fastidious organisms. Moreover, only fresh samples can be used in histologic analysis. Our results reemphasize that CSD may be misdiagnosed as neoplasm, and we believe that lymph node excision and histologic analysis are critical for accurate diagnosis.

We found that 13 *Bartonella*-positive patients (4.2%) had concurrent disease; 10 had mycobacteriosis (Figure 2), and 3 had neoplasm. These patients were older than those with CSD alone. However, neoplasm and mycobacterial infection was less common in patients with CSD than in those without CSD ($p = 0.014$, $n = 181$ patients). In the only report of coincidental CSD and neoplasm, Ridder et al. found 2 patients with squamous cell carcinoma and 2 patients with malignant B-cell lymphoma on the basis of high antibody titers to *B. henselae* (*1*). A high prevalence of *B. henselae*–specific antibodies in HIV-positive patients with generalized lymphadenopathy and patients with non-Hodgkin lymphoma has also been reported (*37*). Explanations for such associations are unknown, and the frequency of asymptomatic patients with CSD is not known. One may speculate that *Bartonella* infections produce more symptoms in patients with HIV infections, mycobacterial infections, or neoplasm or cause chronic infection in such cases.

In conclusion, lymph node excision and testing by histologic analysis are critical in detecting malignant processes and mycobacterial infections, even in patients found to have CSD by PCR. A diagnosis of CSD does not preclude other concurrent diseases, and their presence should routinely be tested by histologic analysis. In addition to testing pus samples or serologic analysis, biopsy specimens should be examined by a histologist, as recently proposed for patients with lymphadenopathy (*8*,*38*,*39*). Our study also demonstrates the advantage of specific target gene amplification compared with 16S rDNA gene amplification. Moreover, physicians should be aware that CSD can occur concurrently with neoplasm and mycobacteriosis, especially in adults >49 years of age.

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Periurban *Trypanosoma cruzi*–infected *Triatoma infestans*, Arequipa, Peru

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In Arequipa, Peru, vectorborne transmission of Chagas disease by *Triatoma infestans* has become an urban problem. We conducted an entomologic survey in a periurban community of Arequipa to identify risk factors for triatomine infestation and determinants of vector population densities. Of 374 households surveyed, triatomines were collected from 194 (52%), and *Trypanosoma cruzi*–carrying triatomines were collected from 72 (19.3%). Guinea pig pens were more likely than other animal enclosures to be infested and harbored 2.38× as many triatomines. Stacked brick and adobe enclosures were more likely to have triatomines, while wire mesh enclosures were protected against infestation. In human dwellings, only fully stuccoed rooms were protected against infestation. Spatially, households with triatomines were scattered, while households with *T. cruzi*–infected triatomines were clustered. Keeping small animals in wire mesh cages could facilitate control of *T. infestans* in this densely populated urban environment.

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, causes more deaths in the Americas than any other parasitic disease (*1*). *T. cruzi* is carried in the gut of bloodsucking triatomine insects (Hemiptera, Reduviidae), and the parasite is usually transmitted to humans when the vector's feces enter the host through the insect bite or mucous membranes (*2*). *Triatoma infestans* is the principal vector of *T. cruzi* in the southern cone of South America and the sole vector in southern Peru. It is a highly synanthropic insect found most often in poor, rural households (*3*,*4*). However, in Arequipa, a city of 850,000 inhabitants in the arid highlands of southern Peru, *T. infestans* and *T. cruzi* have become periurban and urban problems.

Since 1991, *T. infestans* has been the target of an elimination program known as the Southern Cone Initiative (*5*). Member countries of this initiative have controlled or eliminated transmission of Chagas disease by spraying households with pyrethroid insecticides (*6*–*9*). In 2002, the Arequipa Regional Ministry of Health began a spray-based vector control program after an infant in a periurban community died from acute Chagas disease. This initiative, in contrast to those in other parts of the southern cone, is concentrated in and around the city rather than in rural areas. Novel measures may be necessary to prevent vector reinfestation after insecticide application in densely populated environments.

Urbanization of *T. infestans* has been observed elsewhere in South America (*10*–*12*), and other Chagas disease vectors have been observed in cities (*13*,*14*). Nevertheless, little is known about the epidemiology of Chagas disease transmission in and around cities. To tailor vector control strategies for the urban setting, we conducted a study to identify determinants of triatomine infestation and population density in a periurban community of Arequipa. We also examined triatomines for *T. cruzi* to gain a better understanding of the spatial distribution of potential Chagas disease transmission in the community.

Methods

Study Area and Population

Arequipa is located at an elevation of 2,300 m at the foot of an active volcano (16.44° S, 71.59° W). The area is arid; rain is scarce and falls almost exclusively from December through February. Santa Maria de Guadalupe

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and Alto Guadalupe (hereafter referred to together as Guadalupe) are 2 of hundreds of communities located on hillsides on the outskirts of Arequipa (Figure 1). The communities are *pueblos jovenes*, settlements built by displaced families, many of whom migrated from rural areas to the city out of economic necessity after agrarian reform in 1969. Migrants relocated to *pueblos jovenes* in Arequipa in even greater numbers to escape terrorism from 1980 to 1995 (*15*). In preliminary analyses of survey data from 1,444 schoolchildren living in Guadalupe and surrounding communities, 71 (4.9%) had serologic evidence of *T. cruzi* infection (N. Bowman, pers. comm.). The community of Guadalupe consists of 397 dwellings that house ≈2,550 people in an area of 14.1 ha (2,800 households/km^2). Typical households consist of a human dwelling (bedrooms, kitchens, living rooms, and storage rooms) plus an enclosed yard. Roofs of the human dwellings are fully stuccoed or of corrugated metal. Walls consist of a wide variety of materials including *sillar*, a white, porous rock of compounded volcanic ash. Most yards share stone walls with neighboring households, though some back directly up against the basalt (a volcanic stone) rubble of the steep hillside. Neither community underwent systematic insecticide application before this study.

Figure 1. High density of homes in the periurban community of Guadalupe, Arequipa, Peru, November 2004.

Study Design

The entomologic survey was conducted in coordination with the first round of household insecticide application by the Arequipa Ministry of Health Vector Control Program, from November 15 to December 8, 2004. Ministry of Health personnel sprayed each house and all peridomestic structures with deltamethrin powder suspended in water at a rate of 25 mg/m^2 (K-othrine, Bayer, Lima, Peru). After insecticide application, 2 trained triatomine collectors systematically searched each room of the human dwelling, animal enclosure, and remaining peridomestic area for a total of 1 person-hour. Because pilot studies showed marked variation in vector infestation and density within dwellings, data were collected at the level of individual rooms and animal enclosures. An adult from each household responded to a structured questionnaire regarding insecticide usage, cleaning practices, and potential triatomine hosts in each room of the dwelling and each animal enclosure. A collector recorded all construction materials used for each site. Household position was determined with a handheld global positioning system unit with an accuracy of 10 m (Garmin Corporation, Olathe, KS, USA). The protocol was reviewed by the Centers for Disease Control and Prevention's institutional review board.

Triatomines captured from each site were stored separately on ice packs until processing at the National University of San Agustin. Vectors were counted by site, stage, and sex (for adults). Live and moribund fifth instar and adult triatomines were examined for *T. cruzi* consecutively for each site until 1 positive insect was found, 10 negative insects had been examined, or all available insects had been examined, whichever came first. The sampling scheme was designed to detect *T. cruzi* in each site of collection with 80% power if ≥20% of insects were infected. We followed the procedures for examining triatomines for *T. cruzi* outlined in Gürtler et al. (*16*). Briefly, intestinal contents of the insects were extracted by applying pressure to the lower abdomen of the triatomine with forceps. Extracted material was then diluted in 1 drop of saline solution and examined under a microscope at 400× magnification.

Data Analysis

Two outcome variables were examined: *T. infestans* presence (a binary outcome) and *T. infestans* population density as estimated by the number of insects captured in 1 person-hour (a continuous count outcome). Each outcome was examined separately for rooms in human dwellings and animal enclosures. In univariate analyses, associations between triatomine presence and independent variables were evaluated with the χ^2 test for binary variables and Kruskal-Wallis trend test for ordinal and continuous variables. All variables with p value <0.20 in univariate analyses, as well as other likely confounders, were considered in multivariate analyses (*17*). Multivariate models were fit with generalized estimating equations (GEEs). A spatial variogram was used to guide selection of a correlation structure for the GEE analysis (*18*). In the absence of correlation among observations from adjacent households, an exchangeable correlation structure was assumed to adjust confidence intervals for repeated observations from the same household. Nonsignificant variables were dropped sequentially from the multivariate models on the basis of their Wald scores. Analyses were performed

in Stata version 8 (StataCorp LP, College Station, TX, USA) and R version 2.1 (http://www.r-project.org).

Analyses of estimated triatomine density were limited to infested rooms and animal enclosures. To compare the mean number of vectors captured during each timed search, we used zero-truncated negative binomial regression, a method appropriate for analyses of count data in which observations of zero are excluded (*19*). Because the data were overdispersed, the zero-truncated negative binomial distribution fit the data better than the zero-truncated Poisson distribution based on the likelihood-ratio test. GEE methods for zero-truncated negative binomial regression are not available. Therefore, for households with >1 infested room or animal enclosure, 1 site only, selected at random, was included in each analysis to maintain independence of observations. All variables with $p<0.20$ in univariate analyses and other likely confounders were considered for inclusion in a multivariate models of the same type. Analyses were performed in Stata version 8.0.

The spatial K function of Ripley was used to test for spatial clustering of infested households (*20*). Conceptually, the K function measures the expected number of households within a set distance of any given household. The difference between the K function that summarized spatial distribution of *T. infestans*–positive households and the K function that summarized the distribution of *T. infestans*–negative households was calculated. A difference in K functions of greater than zero suggests spatial clustering of positive households (*21*). The analysis was repeated at 30 spatial scales from 10 to 300 m, and for each spatial scale, 99% tolerance limits around the observed difference in K functions for positive and negative households were determined through simulation (*21*,*22*). Clustering of households with *T. cruzi*–positive *T. infestans* was assessed in the same manner. A weighted version of the spatial K function was used to test for clustering of the number of triatomines caught in each house (*23*).

Results

Of 397 households in Guadalupe, 374 (94.2%) were sprayed and surveyed, and 194 (52.0%) were infested with triatomines (Figure 2). Seventy-two households (19.3%) harbored triatomines infected with *T. cruzi.* Triatomines and *T. cruzi*–infected triatomines were present in human dwellings and peridomestic areas. Of 1,424 rooms in human dwellings surveyed, 241 (17%) were infested with vectors and 54 (3.8%) contained *T. cruzi*–infected triatomines. Of 803 animal enclosures, 107 (13%) were infested with vectors and 31(3.9%) had insects infected with parasites. A total of 5,398 triatomines were captured, 2,270 in human dwellings and 3,128 in peridomestic areas. The colonization indices (number of sites with triatomine nymphs/number of sites with triatomines) for rooms and animal enclosures were 76% and 93%, respectively.

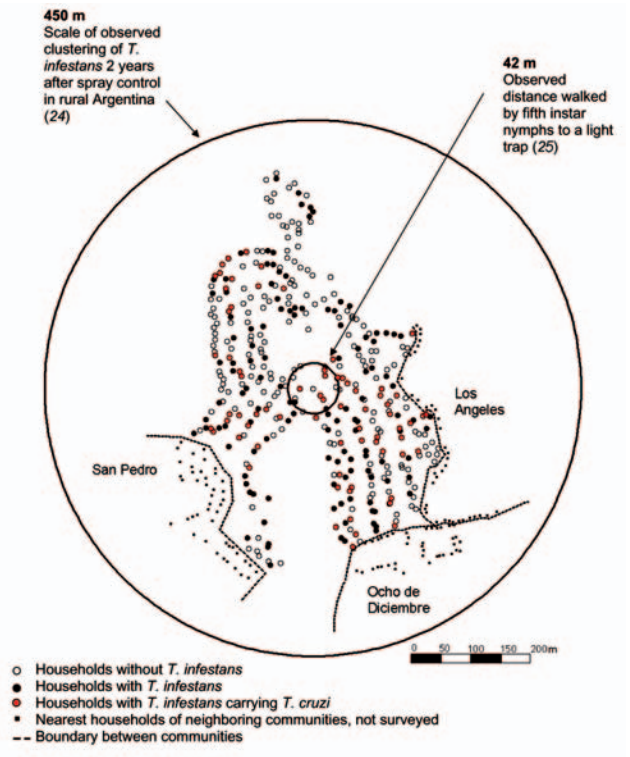

Figure 2. Map of households with *Triatoma infestans* and *Trypanosmona cruzi*–infected *T. infestans* in Guadalupe, a periurban community of Arequipa, Peru. Concentric circles are drawn around a house near the center of Guadalupe and represent parameters of *T. infestans* dispersal observed in rural areas (*24*,*25*). The nearest houses of neighboring communities are included for reference.

At the time of the survey, 263 (70.0%) households in Guadalupe kept domestic animals. We recorded 1,700 guinea pigs, 1,295 rabbits, 819 chickens, 469 sheep, 358 dogs, 135 cats, 126 turkeys, 70 cows, and 34 pigs. Stuccoed enclosures generally housed guinea pigs (29 of 34, correlation coefficient 0.30), chicken-wire enclosures housed rabbits (54 of 134, correlation coefficient 0.18), and adobe enclosures housed sheep (15 of 39, correlation coefficient 0.17). Other types of enclosures contained a range of animals, but none predominated (all correlation coefficients <0.15). Most animals, including large animals, were kept in enclosures on the roof or in the yard of the household; only 66 households (18.7%) allowed some companion animals to sleep inside at night.

T. infestans in Animal Enclosures

Several potential risk factors for triatomine infestation in animal enclosures were identified in univariate analyses (Table 1). In the multivariable logistic model, wire mesh

enclosures were one fifth as likely to be positive than were enclosures of all other materials. Enclosures built wholly or partially of stacked brick or adobe were significantly more likely to be infested than other enclosures. Mortar between units of stone or brick did not significantly reduce the likelihood of infestation. Guinea pig enclosures were 1.69 times as likely to harbor vectors than were other enclosures; chicken enclosures were significantly less likely to be infested than were other enclosures. All 34 fully stuccoed enclosures were negative for *T. infestans* and were omitted from multivariate analyses.

Analyses of triatomine population density included 76 infested enclosures, each from a different household. The average number of insects caught per enclosure was 21.9. Enclosures with guinea pigs had an average of 33.9 insects; wire mesh enclosures averaged 6.7 insects (Table 2). In the multivariate zero-truncated negative binomial model, the presence of guinea pigs was associated with a 2.4-fold increase in estimated triatomine density, and wire mesh enclosures were estimated to harbor only 9% as many triatomines as enclosures of any other material. Some materials in which insect collection was difficult showed lower vector densities.

T. infestans in Rooms of the Human Dwelling

In the multivariate logistic model, the relative odds of infestation in rooms of the human dwelling increased multiplicatively by 1.6 for each additional person sleeping in a room (Table 3). Rooms in which an animal slept were nearly twice as likely as rooms without animals to be positive. Likelihood of being infested was less than one third for fully stuccoed rooms, 1.6 times greater for rooms built with mortared *sillar,* and 1.8 times greater for those built with mortared brick. An infested guinea pig enclosure and yard were both associated with infestation inside the human dwelling.

A random selection of 156 infested rooms, each from a different household, was included in the analysis of vector density (Table 4). Rooms had significantly lower estimated vector densities than did animal enclosures (Kruskal-Wallis p = 0.0001); the overall average number of insects collected in rooms was 8.9. In the multivariate model, the number of insects captured increased by 42% for each person sleeping in the room. Rooms in which animals, mainly dogs and cats, slept had an estimated 5.2 times as many insects as rooms without animals. Rooms with fully stuccoed or adobe walls had significantly fewer triatomines than rooms made of other materials. The density of vectors in rooms of mortared brick, basalt, or *sillar* was not significantly different from that in other rooms after controlling for covariates.

Spatial Analysis

Triatomine-infested households were not significantly clustered at any of the spatial scales examined (Figure 3A). We saw no evidence of clustering in the estimated population density of triatomines across the study site. Households with triatomines infected with *T. cruzi*, however, were significantly more clustered than households without infected insects. The difference in K functions exceeded 99% tolerance limits at all but 1 spatial scale (20 m) from 10 to 140 m (Figure 3B).

Discussion

Elimination of *T. infestans* from Arequipa may be impeded by the ease with which the vector can return to sprayed households in the densely populated urban environment. Just 2 years after blanket deltamethrin spraying

Table 1. Risk factors for *Triatoma infestans* in animal enclosures in a periurban community of Arequipa, Peru*

Risk factor	% of enclosures (n = 803)	Univariate analysis		Multivariate analysis†	
		OR (95% CI)	p value	Adjusted OR (95% CI)	p value
Animal host					
Guinea pig	24	1.54 (0.95–2.4)	0.057	1.69 (1.05–2.74)	0.031
Rabbit	23	1.48 (0.91–2.4)	0.088	1.52 (0.93–2.49)	0.099
Chicken	18	0.28 (0.12–0.62)	0.001	0.36 (0.16-0.80)	0.012
No. animals (mean)‡	5.5		0.29§		
Material					
Wire mesh only	17	0.17 (0.04–0.46)	0.008	0.18 (0.06–0.53)	0.002
Unmortared brick	19	2.96 (1.84–4.70)	0.0001	2.02 (1.23–3.29)	0.005
Adobe	5	2.73 (1.19–5.88)	0.005	2.52 (1.18–5.39)	0.017
Mortared brick, *sillar*, or basalt	12	0.50 (0.19–1.11)	0.08	0.50 (0.21–1.17)	0.11
Unmortared *sillar*‡	30	1.69 (1.08–2.62)	0.14		
Stucco¶	4	0.00 (0.00–0.70)	0.02		
Insecticide use‡	15	1.09 (0.59–1.85)	0.77		

*OR, odds ratio; CI, confidence interval.
†Models were fit with generalized estimating equations appropriate for repeated measures in households.
‡Dropped from multivariate model.
§Kruskal-Wallis trend test for continuous variables.
¶Fully stuccoed enclosures were omitted from the multivariate model because none were positive for *T. infestans*.

Table 2. Determinants of population density of *Triatoma infestans* in animal enclosures in infested households of a periurban community of Arequipa, Peru*

Risk factor	Mean no. triatomines captured (range)	Univariate zero-truncated negative binomial regression		Multivariate zero-truncated negative binomial regression	
		Ratio (95% CI)	p value	Adjusted ratio (95% CI)	p value
Animal host					
Guinea pigs	33.9 (1–343)	2.25 (0.9–5.8)	0.09	2.38 (1.0–5.7)	0.05
No animals present	2.6 (1–7)	0.08 (0.02–0.32)	<0.01	0.15 (0.04–0.57)	<0.01
Chickens†	28.2 (1–79)	1.38 (0.23–8.4)	0.70		
Turkeys†‡	6.0 (1–15)	0.18 (0.02–1.5)	0.11		
No. animals†§		1.0 (0.92–1.22)	0.65		
Material					
Wire mesh only	6.7 (1–10)	0.21 (0.02–1.75)	0.15	0.09 (0.01–0.6)	0.02
Unmortared basalt	11.8 (1–109)	0.38 (0.15–0.96)	0.04	0.35 (0.15–0.80)	0.01
Mortared brick, basalt, and *sillar* only	6.5 (3–15)	0.20 (0.03–1.3)	0.09	0.21 (0.04–1.1)	0.06
Unmortared brick†	23.3 (1–90)	1.13 (0.46–2.8)	0.80		
Adobe†	21.0 (1–112)	0.94 (0.22–4.0)	0.94		
Insecticide use†	29.8 (1–112)	1.1 (0.45–2.7)	0.80		

*Triatomine density was estimated by timed capture of insects by trained collectors. OR, odds ratio; CI, confidence interval.
†Dropped from the multivariate model.
‡1 turkey enclosure, which contained 598 *T. infestans*, was considered an outlier and left out of analysis.
§ No mean is given for continuous variables.

in a rural community in Argentina, *T. infestans* was found in sites clustered within 450 m of a putative source population (*24*). If the range of *T. infestans* redispersion is similar in Guadalupe, a single residual population of vectors would put every household in the community, as well as many households in 3 neighboring communities, at risk for vector reinfestation. *T. infestans* nymphs can walk ≥42 m (*25*) and can easily climb across or through crevices in the stone walls separating the densely packed houses of Guadalupe (2,800 households/km^2). In the rural Argentina site, with 42 households/km^2 (*26*), vector redispersion was thought to be through flight of adult insects (*24*). *T. infestans* adults usually fly only under specific conditions (*25*). In densely populated urban and periurban sites, walking is likely to be the principal mode of redispersion, and reinfestation is likely to be much more rapid than in rural settings.

Vector reinfestation typically begins in the peridomestic environment, where domestic animals are kept (*27*). The guinea pig, a staple source of protein in the Andes for thousands of years (*28*), and reportedly a reservoir of Chagas disease in Peru (*29–31*), is the most numerous domestic animal in Guadalupe. As our data demonstrate, guinea pig presence is also a determinant of peridomestic *T. infestans* infestation. Enclosures where guinea pigs were housed were more likely to be infested and, when infested, harbored twice as many vectors as other enclosures. Schofield hypothesizes that *T. infestans* population growth slows when host protective behavior, such as scratching and swatting, limit the insects' ability to complete a blood meal (*32*,*33*). Incompletely fed triatomines have delayed

Table 3. Risk factors for *Triatoma infestans* in rooms of human dwellings in a periurban community of Arequipa, Peru*

Risk factor	% of rooms (N = 1,424)	Univariate analysis†		Multivariate logistic regression†	
		OR (95% CI)	p value	Adjusted OR (95% CI)	p value
Host					
No. persons sleeping in room			<0.001‡	1.63 (1.48–1.79)	<0.001
Animal sleeping in room	5.1	2.79	<0.001	1.90 (1.10–3.28)	0.021
Peridomestic infestation					
Guinea pig enclosure	9.1	1.70 (1.07–2.63)	0.014	2.23 (1.30–3.82)	0.004
Yard	11.7	1.41 (0.92–2.12)	0.09	2.10 (1.27–3.46)	0.004
Sheep enclosure§	5.3	2.29 (1.31–3.90)	0.001		
Material					
Mortared brick	19.0	2.39 (1.73–3.30)	<0.001	1.76 (1.15–2.71)	0.01
Mortared *sillar*	20.2	2.00 (1.44–2.76)	<0.001	1.60 (1.04–2.47)	0.033
Fully stuccoed	41.7	0.25 (0.17–0.38)	<0.001	0.27 (0.17–0.44)	<0.001
Unmortared *sillar*§	26.5	1.21 (0.81–1.78)	0.32		
Insecticide use§	43.8	0.91 (0.68–1.22)	0.53		

*OR, odds ratio; CI, confidence interval.
†Models were fit with generalized estimating equations appropriate for repeated measures in households.
‡Kruskal-Wallis trend test for continuous variable.
§Dropped from the multivariate model.

Table 4. Determinants of population density of *Triatoma infestans* in rooms of human dwellings in a periurban community of Arequipa, Peru*

Risk factor	Mean no. triatomines captured (range)	Univariate zero-truncated negative binomial regression		Multivariate zero-truncated negative binomial regression	
		Ratio (95% CI)	p value	Adjusted ratio (95% CI)	p value
Host					
Each person sleeping in room†		1.58 (1.29–1.93)	<0.001	1.42 (1.16–1.72)	0.001
Animal sleeping in room	22.12 (1–124)	7.34 (2.20–24.54)	0.001	5.23 (1.56–17.47)	0.007
Material					
Fully stuccoed walls and ceiling	3.03 (1–20)	0.065 (0.03–0.16)	<0.001	0.11 (0.04–0.31)	<0.001
Adobe	1.73 (1–4)	0.055 (0.013–0.24)	<0.001	0.15 (0.032–0.73)	0.019
Mortared brick, *sillar*, or basalt	12.62 (1–124)	2.25 (1.08–4.71)	0.03	0.80 (0.35–1.84)	0.599‡
Unmortared brick, *sillar*, or basalt§	11.85 (1–103)	1.58 (0.65–3.87)	0.313		
Insecticide use§	9.74 (1–124)	1.20 (0.32–2.44)	0.808		

*Triatomine density was estimated by timed-capture of insects by trained collectors. CI, confidence interval.
†No mean is given for continuous variables.
‡The variable describing mortared brick, *sillar*, and basalt was left in the final model because it represents a possible intervention and was therefore a principal variable of interest. Removing the variable does not greatly affect the estimates for the other parameters or the significance level of those estimates.
§Dropped from the multivariate model.

molting (*34*) and are more likely to migrate (*25*,*35*); vector population sizes thereby decrease without an increase in insect deaths (*33*). Compared with other animals, guinea pigs may be less able to repel feeding vectors. Their habit of pressing against enclosure walls may also facilitate triatomine feeding and increase vector population growth.

Chickens are associated with infestation and increased triatomine density in rural settings, where they typically range and roost freely (*4*,*36*). Cecere et al. suggest that confining chickens might reduce triatomine populations (*36*); in Guadalupe, space constraints force most households to keep chickens enclosed. The result seems to be a decrease in the importance of chickens as hosts for *T. infestans*. Chickens hunt triatomines by sight (*34*) and may be more able to detect and catch insects from the walls of urban enclosures than from roosting materials in rural settings.

The materials used to build animal enclosures were stronger predictors of *T. infestans* presence than were the type of animals housed in the enclosure. Many materials cheaply or freely available in Arequipa, such as unmortared brick, *sillar*, and basalt, provide ample refuge for insects. Adobe was less common but was also associated with an increased risk for infestation. Fully stuccoed enclosures were never infested in Guadalupe but are costly to build. Replacing small animal enclosures of brick, *sillar*, basalt, and adobe with inexpensive wire mesh structures, which are refractory to triatomine colonization, may be the most feasible intervention to slow or prevent vector reinfestation.

The presence and density of triatomines in rooms of human dwellings are critical determinants of the risk for Chagas disease transmission to humans (*4*,*36*,*37*). In Guadalupe, the number of persons who slept in a room was the principal predictor of infestation and a determinant of vector population density. Simple interventions to decrease domestic triatomine populations in rural areas, such as keeping animals outside at night (*38*) and improving roofing materials (*39*), will likely have limited effect in Guadalupe. Although the presence of companion animals in rooms at night was associated with a 5-fold increase in vector density, animals were allowed to sleep inside in only 5% of rooms. Nearly all roofs in the community were of corrugated metal or other materials that do not provide refuge for triatomines. The only housing intervention that is likely to have a substantial effect against domiciliary triatomines in Guadalupe is completely stuccoing rooms. Schofield and Marsden showed that completely stuccoing a house could eliminate *T. infestans* from the human dwelling within 3 years (*40*). In Guadalupe, stuccoed rooms were only a fourth as likely to be infested and harbored a tenth the population of vectors compared with rooms of other materials. However, stuccoing must be complete to be effective; rooms in which mortar was used to fill the gaps between brick, *sillar*, and basalt were significantly more likely to be infested with the vector than rooms of other types.

Our study had several limitations. Triatomine collection was dependent on the excito-repellant effect of deltamethrin spray. In some materials, especially the unmortared basalt of the hillside, insecticide did not reach all refuges of triatomines, and our vector collections may have been incomplete. *T. infestans* can survive for many months without feeding, and insect population density may be more influenced by past, rather than present, host populations (*4*). We did not have information to evaluate the effect of previous animal populations on size of *T. infestans*

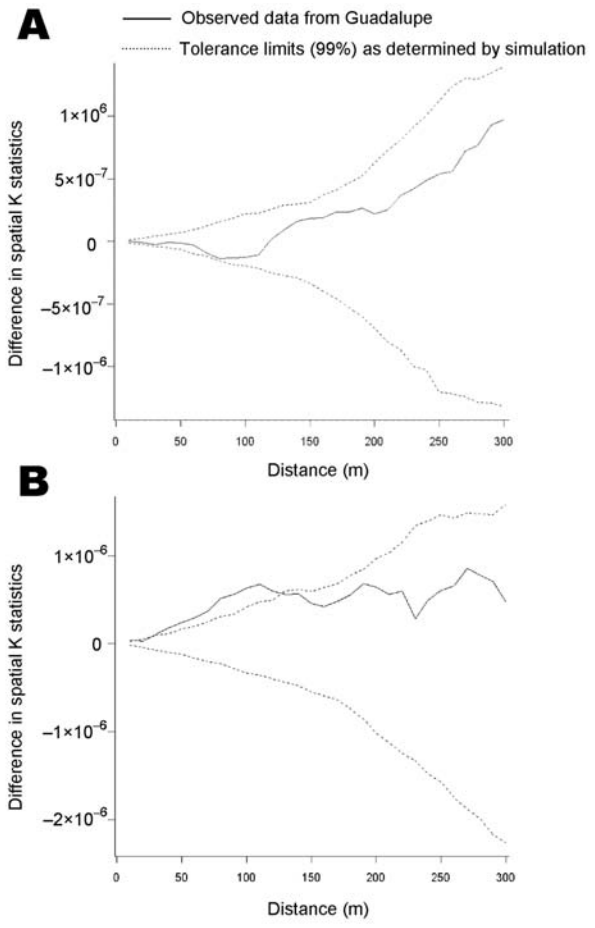

Figure 3. Clustering analysis of A) houses with *Triatoma infestans* and B) houses with *Trypanosoma cruzi*–infected *T. infestans* in a periurban community of Arequipa, Peru.

populations at the time of spraying. Identification of households with triatomines infected with *T. cruzi* was limited by the number of insects captured at each site of collection, and the number of insects examined varied between households. The power of the analyses of estimated vector densities was diminished because we considered only 1 enclosure and room per household to maintain independence of observations.

Spatial analysis suggests that while the vector is distributed across Guadalupe, Chagas disease transmission is likely to be clustered. Households with *T. cruzi*–infected triatomines showed significant clustering. Many aspects of the complicated periurban ecology of reservoir, vector, and parasite populations could lead to spatial clustering of *T. cruzi* without clustering of its vector, but the most parsimonious explanation is a basic difference in the speed of vector and parasite dispersion. Guadalupe is a young community; 81% of households were constructed in the past 20 years. While triatomines may have had sufficient time to infest and colonize most suitable habitats in Guadalupe and may be considered endemic, *T. cruzi* may still be spreading from 1 or multiple points of introduction in a more epidemic fashion.

Many communities similar to Guadalupe are awaiting insecticide application. Acute cases of Chagas disease have been reported from communities in different parts of the city (Arequipa Regional Ministry of Health, unpub. data), though transmission of *T. cruzi* in these areas is likely focal. Timely, coordinated insecticide application is imperative to control Chagas disease in southern Peru and must be accompanied by effective surveillance for vector reinfestation. Improvement of peridomestic small animal enclosures with materials refractory to triatomine infestation could greatly increase the likelihood of eliminating the vector from the city.

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Genomic Signatures of Human versus Avian Influenza A Viruses

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Position-specific entropy profiles created from scanning 306 human and 95 avian influenza A viral genomes showed that 228 of 4,591 amino acid residues yielded significant differences between these 2 viruses. We subsequently used 15,785 protein sequences from the National Center for Biotechnology Information (NCBI) to assess the robustness of these signatures and obtained 52 "species-associated" positions. Specific mutations on those points may enable an avian influenza virus to become a human virus. Many of these signatures are found in NP, PA, and PB2 genes (viral ribonucleoproteins [RNPs]) and are mostly located in the functional domains related to RNP-RNP interactions that are important for viral replication. Upon inspecting 21 human-isolated avian influenza viral genomes from NCBI, we found 19 that exhibited ≥1 species-associated residue changes; 7 of them contained ≥2 substitutions. Histograms based on pairwise sequence comparison showed that NP disjointed most between human and avian influenza viruses, followed by PA and PB2.

Pandemic influenza A virus infections have occurred 3 times during the past century; the 1957 (H2N2) and 1968 (H3N2) pandemic strains emerged from a reassortment of human and avian viruses (*1*). Recently, all 8 genome segments from the 1918 (H1N1) influenza A virus were completely sequenced. The results indicate that the 1918 pandemic virus may not have emerged by a reassortment of avian and human virus as did the 2 other pandemic strains. Although the 1918 H1N1 is not considered an avian virus, it is the most avianlike of all mammalian influenza viruses (*2*,*3*). The recent circulation of highly pathogenic avian H5N1 viruses in Asia from 2003 to 2006 has caused ≥90 human deaths and has raised concern about a new pandemic (*4*). Therefore, we need to understand what genetic variations could render avian influenza virus capable of becoming a pandemic strain. Genomewide comparison of human versus avian influenza A viruses would show the evolutionary similarities and differences between them and thus provide information for studying the mechanism of influenza viral infection and replication in different host species.

Although many research efforts have focused on the molecular evolution of specific genes of influenza viruses, comprehensive comparisons among the nucleotide sequences of all 8 genomic segments and among the 11 encoded protein sequences have not been extensively reported. In this study, we used several computational approaches for finding specific genetic signatures characteristic of human and avian influenza A viral genomes. We subsequently validated the robustness of those signatures with human and avian protein sequences downloaded from Influenza Virus Resources at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih. gov/genomes/FLU/FLU.html).

Materials and Methods

Clinical Isolates

Throat swabs from patients with influenzalike syndromes were collected from the Clinical Virology Laboratory, Chang Gung Memorial Hospital. The specimens were inoculated in MDCK cells. Typing for influenza A virus was then performed with immunofluorescent assay by type-specific monoclonal antibody (Dako, Cambridgeshire, UK). Subtyping was conducted by reverse transcription (RT)–PCR with subtype-specific primers.

Sequence Analysis

The RT-PCR product was purified by using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). The nucleotide sequence was determined with an

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automated DNA sequencer. Sequence editing and processing were performed with Lasergene, version 3.18 (DNASTAR, Madison, WI, USA). Multiple sequence alignment was performed with ClustalW version 1.83 (ftp://ftp.ebi.ac.uk/pub/software/unix/clustalw). Global sequence comparison that yielded pairwise sequence identities used in histogram analysis was done with the program Needle in the EMBOSS package (*5*). Amino acid sequences were translated from coding sequences and aligned by BioEdit (*6*). An entropy value was defined at an aligned amino acid position according to the formula $\Sigma P_i * log(P_i)$, in which i is the observed probability for each of the 20 amino acids (aa) (*7*). A graphic tool was developed in Java for displaying the entropy plot used in this work. All amino acid numberings are based on influenza virus A/Puerto Rico/8/1934 (PR8).

Sequences Used in Study

To show the host-associated amino acid signatures, we retrieved full genome sequences (as of August 22, 2005) from the genome browser at Influenza Sequence Database (ISD) (*8*). To differentiate between avian and human influenza viruses, we excluded human-isolated avian influenza viruses from the human dataset and examined those sequences separately. Altogether, we had 95 avian and 306 human influenza viral genomes, henceforth termed "primary dataset." All 11 viral proteins encoded by the 8 genomic RNA segments were compared: PB2, PB1, PB1-F2, PA, HA, NP, NA, M1, M2, NS1, and NS2.

Avian influenza viruses from human influenza patients were separately retrieved from NCBI as well as from ISD. Altogether, we had 417 protein sequences from 60 avian influenza strains, in which 21 strains contain sequences (full or nearly full length) from all 8 genomic RNA segments.

For validating the signatures obtained from analyzing the primary dataset, we further retrieved 15,785 human or avian influenza A viral protein sequences from NCBI's Influenza Virus Resources. Details for the sequences used can be found in online Appendix, Supporting Materials and Methods (available from http://www.cdc.gov/ncidod/EID/vol12no09/06-0276.htm#app), as well as in online Appendix Table 1 (http://www.cdc.gov/ncidod/EID/vol12no09/06-0276_appT1.htm) and online Appendix Table 2 (http://www.cdc.gov/ncidod/EID/vol12no09/06-0276_appT2.htm). Eleven Taiwanese genomes produced in this work have been deposited in GenBank with accession numbers DQ415283 through DQ415370.

Results

Differing Amino Acid Residues

Using previously described methods (*7*), we separately calculated an entropy value for every aligned amino acid position for 95 avian influenza viruses and 306 human influenza viruses. Those amino acid residues with an entropy value between 0 and –0.4 for both the human and avian strains were identified as most highly conserved. We chose this entropy threshold on the basis of the entropy value –0.379, calculated at position 627 of PB2 for the 95 avian viruses. This widely reported, species-associated residue is highly conserved; it has E (Glu) in 83 and K (Lys) in 12 avian isolates and Lys in all 306 human isolates. We then selected those conserved positions with distinct amino acid residues between human and avian influenza viruses as potential host-associated signatures. An entropy plot for identifying such signature residues for avian versus human influenza virus NP segments is shown in Figure panel A. In each aligned position, we placed an avian consensus residue on top and a human consensus at the bottom. For example, the entropy value is zero at amino acid position 283 for both avian and human strains, in which all 95 avian influenza viruses contain L (Leu), whereas all 306 human influenza viruses contain P (Pro). The other 2 residues with zero entropy value in avian and human viruses are located at position 55 of PA, in which we have D (Asp) in avian viruses and N (Asn) in human viruses, and position 121 of M1, in which we have T (Thr) in avian and A (Ala) in human viruses. Entropy plots for all 11 influenza viral proteins can be found in online Appendix Figure 1 (http://www.cdc.gov/ncidod/EID/vol12no09/06-0276_appG1.htm).

Figure panel B shows a genomewide view of the entropy plots for 11 influenza A viral proteins. The amino acid sequences of hemagglutinin (HA), with an average entropy value of –0.524 within avian viruses and –0.158 within human viruses, exhibit much more diversity than other open reading frames (ORFs). PB2, PB1, PA, NP, and M1, on the other hand, are more conserved (i.e., they have less negative entropy values).

In addition to the previously mentioned 3 positions with distinct amino acid residues between avian and human strains, we found 225 additional positions with nearly distinct amino acid residues, with their computed entropy values less negative than –0.4 in both the 306 human and 95 avian strains that we analyzed. To assess the robustness of those 228 residues used in differentiating human from avian influenza viruses, we further examined 15,785 influenza A protein sequences from NCBI. After validation, 52 positions still showed an entropy value less negative than –0.4 and conserved to distinct amino acid residues between human and avian viruses (Table 1). From this entropy analysis, we identified an additional 51 aa positions that may be as important as the well-known position 627 of PB2. We designated these 52 positions as "species-associated" signatures. Among 11 ORFs, NP contains the highest number of such signatures (15 positions),

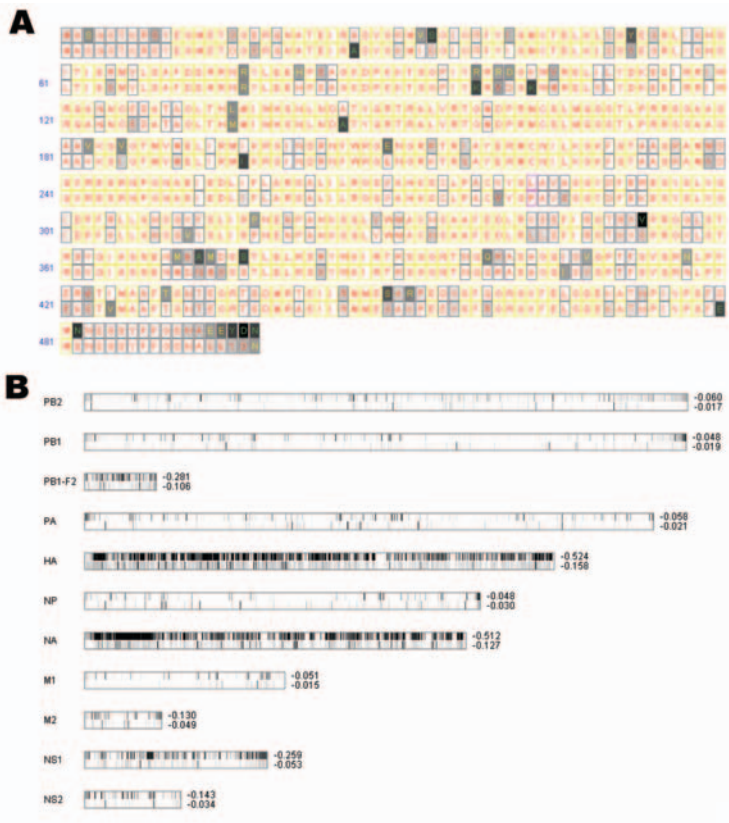

Figure. A) Entropy plot for avian versus human influenza viruses for NP amino acid residues. In each aligned position, we have a consensus residue for 95 avian strains displayed on top and a consensus residue for 306 human strains at the bottom. Completely conserved amino acid positions are filled with white; less conserved amino acids are filled in various gray shadings. Positions in which 1 single residue dominates >90%, <90% but >75%, and <75% are labeled with red, yellow, and green letters, respectively. Yellow rectangles indicate that both human and avian viruses are completely conserved to the same residue; magenta rectangles indicate that avian and human viruses are each completely conserved to a different residue. B) Entropy plots for the entire influenza A viral genome. Each lane displays entropy value distributions of aligned protein sequences for 1 of the 11 viral proteins; the upper half represents 95 avian strains, and the bottom half represents 306 human strains. (PB1-F2 contains fewer strains, as described in Discussion.) Positions completely conserved to a single residue are shown in a white band, while less conserved ones are shown in various gray shadings. The average entropy for the entire segment is shown to the right of these lanes. Entropy values are zero when residues are completely conserved; more negative values indicate more diversity. Alignment size for each protein from top to bottom is 759, 757, 90, 716, 591, 498, 480, 252, 97, 230, and 121.

followed by PA (10 positions), PB2 (8 positions), PB1-F2 (5 positions), M2 (4 positions), M1 (3 positions), PB1 (2 positions), HA (2 positions), NS2 (2 positions), and NS1 (1 position). No signature was found in the NA gene. We also summarized the related functions of those species-associated signatures in Table 1. The complete results of genome scanning and validation can be found in online Appendix Table 3 (http://www.cdc.gov/ncidod/EID/vol12no09/06-0276_appT3.htm) and online Appendix Table 4 (http://www.cdc.gov/ncidod/EID/vol12no09/06-0276_appT4.htm).

Amino Acid Signatures in Human Viruses

We examined how the amino acid sequences varied at those proposed signature positions for avian influenza viruses isolated from humans. At 9 of these 52 positions, residue changes were characteristic of human rather than avian viruses (Table 2). For example, 34 sequences (27 H5N1, 3 H9N2, and 4 H7N7) were available for inspection at position 199 of PB2 (data not shown). Aside from 10 sequences with gaps (sequences did not cover this position), 19 of the remaining 24 still have Ala, which is typical for avian viruses. Five of them (all H5N1), on the other hand, have this residue changed to Ser, which is mostly seen in human viruses. At the well-known position 627 of PB2, 5 sequences had gaps, 22 retained Glu (typical for avian virus), while the other 7 changed to Lys, which is typical for human virus. Among those 7 mutated sequences, 6 were from H5N1 human isolates (A/Hong Kong/483/1997, A/Hong Kong/485/1997, A/Vietnam/1194/ 2004, A/Vietnam/1203/2004, A/Vietnam/3062/2004, and A/Thailand/16/2004), and the other 1 was A/Netherlands/219/2003(H7N7), which was isolated from a fatal human case of pneumonia in the Netherlands (*32*).

To understand how mutations had accumulated within a specific virus, we summarized the amino acid changes for 21 of these avian viruses that contained full or nearly full-length sequences for each segment (Table 3). We found that 19 of 21 strains contained $\geq$1 species-associated amino acid change, and 7 of them contained $\geq$2 substitutions; A/Netherlands/219/2003(H7N7) had the highest count for mutation accumulation (3 positions). Among these 52 species-associated signatures, the mutation combinations at positions PB2 199 and PA 409 were most commonly seen in H5N1 human isolates from Hong Kong in 1997.

RNA Segment 5

Our observation that NP contained the highest number (15 of 52) for species-associated amino acids suggested that NP might serve as a molecular target for differentiation between human and avian influenza A viruses. To indicate such host specificity, or the "genetic boundary"

Table 1. Validated amino acid signatures separating avian influenza viruses from human influenza viruses*

Gene	Position	Avian residues	Human residues	Associated functional domains
PB2	44	**A**(208),S(7)	**S**(831),A(10),L(2)	PB1–1, NP-1 (*9*), MLS (*10*)
	199	**A**(210),S(5)	**S**(842),A(3)	NP-1 (*9*)
	271	**T**(210),A(3),I(1),M(1)	**A**(836),T(6),S(1)	Cap-N (*11*)
	475	**L**(214),M(1)	**M**(839),L(3)	NLS (*12*)
	588	**A**(203),T(6),V(6)	**I**(835),V(3),A(2)	PB1–2, NP-2 (*9*)
	613	**V**(212),A(3)	**T**(816),I(16),A(8),V(1)	PB1–2, NP-2 (*9*)
	627	**E**(196),K(19)	**K**(838),R(2),E(1)	PB1–2, NP-2 (*9*)
	674	**A**(204),S(6),T(2),G(2),E(1)	**T**(836),A(2),I(2),P(1)	PB1–2, NP-2 (*9*)
PB1	327	**R**(147),K(3)	**K**(766),R(66)	cRNA (*13*)
	336	**V**(142),I(8)	**I**(773),V(59)	cRNA (*13*)
PB1-F2	73	**K**(397),R(6),I(1)	**R**(594),K(87),S(1)	ANT3, VDAC1 (*14*), mitochondrial localization (*15*), predicted amphipathic helix (*16*)
	76	**V**(401),A(3)	**A**(625),V(57)	ANT3, VADC1 (*14*), predicted amphipathic helix (*16*)
	79	**R**(369),Q(34),L(1)	**Q**(607),R(75)	ANT3, VADC1 (*14*), predicted amphipathic helix (*16*)
	82	**L**(382),S(22)	**S**(596),L(86)	ANT3, VADC1 (*14*), predicted amphipathic helix (*16*)
	87	**E**(389),G(14),K(1)	**G**(637),E(45)	ANT3, VADC1 (*14*)
PA	28	**P**(213),S(1)	**L**(831),P(9),R(2)	Proteolysis (*17*)
	55	**D**(214)	**N**(836),D(5)	Proteolysis (*17*)
	57	**R**(210),Q(4)	**Q**(829),R(6),L(4),K(2)	Proteolysis (*17*)
	225	**S**(213),C(1)	**C**(829),S(10)	Proteolysis (*17*), NLSII (*18*)
	268	**L**(214)	**I**(827),L(11), P(1)	
	356	**K**(212),X(1),R(1)	**R**(827),K(11)	
	382	**E**(208),D(5),V(1)	**D**(824),E(11),V(2),N(1)	
	404	**A**(214)	**S**(828),A(9),P(1)	
	409	**S**(189),N(24),I(1)	**N**(830),S(7),I(1)	
	552	**T**(213),N(1)	**S**(835),T(1),I(1)	
HA	237	**N**(582),R(49),D(2),H(1),S(1)	**R**(1209),N(12),S(2),D(1),K(1)	
	389	**D**(659),N(20),G(1),Y(1)	**N**(819),D(121)	
NP	16	**G**(356),S(9),D(6),T(2)	**D**(646),G(7)	RNA binding (*19*), BAT1/UAP56 (*20*), MxA (*21*), PB2–1 (*22*)
	33	**V**(355),I(18)	**I**(638),V(15)	RNA binding (*19*), MxA (*21*), PB2–1 (*22*)
	61	**I**(366),M(6),V(1)	**L**(642),I(8)	RNA binding (*19*), MxA (*21*), PB2–1 (*22*)
	100	**R**(360),K(11),V(2)	**V**(619),I(32),A(1),M(1)	RNA binding (*19*), MxA (*21*), PB2–1 (*22*)
	109	**I**(359),V(10),M(2),T(2)	**V**(614),I(34),T(3),A(2)	RNA binding (*19*), MxA (*21*), PB2–1 (*22*)
	214	**R**(352),K(20),L(1)	**K**(640),R(10)	NLS (*23*), CRM1 (*24*), NP-1 (*25*)
	283	**L**(372),P(1)	**P**(643),L(7)	NP-1 (*25*), PB2–2 (*22*)
	293	**R**(371),K(2)	**K**(622),R(28)	NP-1 (*25*), PB2–2 (*22*)
	305	**R**(369),K(4)	**K**(636),R(14)	NP-1 (*25*), PB2–2 (*22*)
	313	**F**(371),I(1),L(1)	**Y**(642),F(8)	NP-1 (*25*), PB2–2 (*22*)
	357	**Q**(368),K(4),T(1)	**K**(644),R(8),Q(1)	NAS (*26*), NP-1 (*25*), PB2–3 (*22*)
	372	**E**(357),D(15),K(1)	**D**(630),E(23)	NAS (*26*), NP-2 (*25*), PB2–3 (*22*)
	422	**R**(373)	**K**(630),R(23)	CTL epitope (*27*), NP-2 (*25*), PB2–3 (*22*)
	442	**T**(372),A(1)	**A**(629),T(23),R(1)	NP-2 (*25*), PB2–3 (*22*)
	455	**D**(373)	**E**(630),D(22),T(1)	NP-2 (*25*), PB2–3 (*22*)
M1	115	**V**(856),I(2),L(1),G(1)	**I**(981),V(9)	
	121	**T**(840),A(19),P(1)	**A**(988),T(2)	
	137	**T**(859),A(1),P(1)	**A**(974),T(12)	
M2	11	**T**(434),I(11),S(2)	**I**(911),T(44)	Host restriction specificities (*28*), ectodomain (*29*)
	20	**S**(471),N(13)	**N**(926),S(29)	Host restriction specificities (*28*). ectodomain (*29*)
	57	**Y**(481),C(1),H(1)	**H**(913),Y(33),R(2),Q(1)	CRAC (*30*), endodomain (*29*)
	86	**V**(378)	**A**(924),V(10),T(4),D(1)	Endodomain (*29*)
NS1	227	**E**(692),G(9),K(1),S(1)	**R**(897),G(5),K(1),E(1)	
NS2	70	**S**(453),G(21),D(1)	**G**(903),S(2)	M1, NEP dimerization domain (*31*)
	107	**L**(468),S(2),F(1)	**F**(777),L(16),S(1)	M1, NEP dimerization domain (*31*)

*Numbers in parentheses in residue columns are the number of sequences yielding the specific amino acid residue; **bold** indicates dominant amino acid residue type.

Table 2. Summary of host-associated amino acid signature changes

Gene	Position	Residue*	H5N1	H9N2	H7N2	H7N7
PB2	199	A(19)	15	3		1
		S(5)	5			
	271	T(23)	20	2		1
		A(1)		1		
	627	E(22)	19	3		
		K(7)	6			1
PB1-F2	73	K(24)	17	2		5
		R(2)	2			
	79	R(24)	17	2		5
		Q(2)	2			
	82	L(21)	19	2		
		S(5)				5
PA	409	S(17)	12	3		2
		N(7)	7			
M2	20	S(34)	31	2	1	
		N(5)				5
NS2	70	S(26)	22	2		2
		G(1)		1		

*Top half displays an avian-specific residue with the count in parentheses and distribution among subtypes, and the bottom half represents a human-specific residue.

between these 2 viruses at the nucleotide level, we performed a pairwise sequence comparison for all 11 ORFs on our 401-genome primary dataset and produced histograms on their computed pairwise identities. In online Appendix Figure 2 (http://www.cdc.gov/ncidod/EID/vol12no09/06-0276_appG2.htm), pairs with 2 sequences of the same host species (human to human, or avian to avian; termed homopairs) and pairs for sequences that cross host species (human to avian, or avian to human; termed heteropairs) are shown. HA and NA genes exhibited considerable sequence differences between strains, with identities as low as 47%. Also noted was a wide spectrum of percent identities (e.g., 55%–95% in the horizontal axis) containing few sequence pairs for these 2 genes. For both of these proteins, some strains from the same species can have identities as low as 50%. However, the ORF of another surface protein, M2 ion channel protein, is relatively conserved (>74% identity for viruses across species). The histograms for the polymerase genes (PB2, PB1, and PA), NP, and M1, on the other hand, are much less varied (mostly <20% variation). In particular, the NP gene was found to exhibit a fairly clear boundary between homopairs and heteropairs, at ≈86%.

Discussion

The glutamic acid residue at PB2 627, which is commonly seen in avian viruses, restricts viral growth in humans and monkeys, but a change to lysine restores virus replication in mammalian cells (*33*). In this study we computed for every amino acid position (distributed in the 11 known influenza viral ORFs) an entropy value that represents how conserved an amino acid residue is at that given position. We found the entropy value –0.379 at 627 of PB2 and therefore used –0.4 as a threshold to discover other amino acid residues that might be potential determinants of

Table 3. Twenty-one avian influenza A viral genomes isolated from humans and their mutations found at 12 host-associated positions within each strain*

		PB2			PB1-F2			PA	M2	NS2	
Strain	Subtype	199	271	627	73	79	82	409	20	70	Mutations
A/Hong Kong/156/1997	H5N1	**S**	T	E	K	R	L	**N**	S	S	2
A/Hong Kong/481/1997	H5N1	A	T	E	K	R	L	**N**	S	S	1
A/Hong Kong/482/1997	H5N1	**S**	T	E	K	R	L	**N**	S	S	2
A/Hong Kong/483/1997	H5N1	A	T	**K**	K	R	L	S	S	S	1
A/Hong Kong/485/1997	H5N1	A	T	**K**	**#**	**#**	**#**	S	S	S	1
A/Hong Kong/486/1997	H5N1	**S**	T	E	K	R	L	**N**	S	S	2
A/Hong Kong/532/1997	H5N1	A	T	E	K	R	L	**N**	S	S	1
A/Hong Kong/538/1997	H5N1	**S**	T	E	K	R	L	**N**	S	S	2
A/Hong Kong/542/1997	H5N1	A	T	E	K	R	L	**N**	S	S	1
A/Hong Kong/1997/1998	H5N1	**S**	T	E	K	R	L	S	S	S	1
A/Hong Kong/212/2003	H5N1	A	T	E	**R**	R	L	S	S	S	1
A/Hong Kong/213/2003	H5N1	A	T	E	**R**	R	L	S	S	S	1
A/Thailand/16/2004	H5N1	A	T	**K**	K	**Q**	L	S	S	S	2
A/Thailand/SP83/2004	H5N1	A	T	E	K	**Q**	L	S	S	S	1
A/Vietnam/1194/2004	H5N1	A	T	**K**	K	R	L	S	S	S	1
A/Vietnam/1203/2004	H5N1	A	T	**K**	K	R	L	S	S	S	1
A/Vietnam/3062/2004	H5N1	A	T	**K**	K	R	L	S	S	S	1
A/Netherlands/219/2003	H7N7	A	T	**K**	K	R	**S**	S	**N**	S	3
A/Guangzhou/333/1999	H9N2	A	**A**	E	**#**	**#**	**#**	S	S	**G**	2
A/Hong Kong/1073/1999	H9N2	A	T	E	K	R	L	S	R	S	0
A/Hong Kong/1074/1999	H9N2	A	T	E	K	R	L	S	S	S	0

*# indicates strains with PB1 RNA encoded into a truncated form of PB1-F2 of only 57 amino acids long. **Boldface** letters represent mutated (human-specific) residues; Roman (nonbold) letters are used for regular avian residue. Note that at position 20 of M2, A/Hong Kong/1073/99 had its residue changed from S to R, where R is still considered a mutation within avian species.

host-cell tropism. Another 51 positions were found to be distinct or nearly distinct between human and avian viruses by this entropy threshold. Most of these (40 of 52) are located in viral ribonucleoproteins (RNPs) (PB2, PB1, PA, and NP), which are essential for viral replication. Taubenberger et al. reported 10 amino acid residues that distinguish human and avian influenza viral polymerases (*3*). Six of them were also identified in this study. The entropy values of the 4 missing ones were also found close to the preset threshold (–0.4). For example, PB2 567 showed a human entropy of –0.039 and avian entropy of –0.490, PB1 375 with human entropy –0.165 and avian entropy –0.693, and PA 100 with human entropy –0.061 and avian entropy –0.406. All 3 positions were eliminated earlier from the stage of analyzing the 401-genome primary dataset. The fourth position, PB2 702, although in the first-round list, marginally failed in the subsequent validation with human entropy –0.057 and avian entropy –0.404.

We proposed a computational approach capable of indicating species-associated signatures in studying human versus avian influenza viral genomes. Although we intended to analyze a comprehensive set of avian versus human influenza A viral genomes, the available sequences are predominated by H5N1 in avian viruses and H3N2 in human viruses. The short supply of sequences other than those 2 subtypes may inevitably cause a certain amount of bias in our results. At the completion of this study, we noticed a recent article by Obenauer et al., who had made 169 newly sequenced avian influenza viral genomes available to GenBank on January 26, 2006 (*34*); these were not included in our analysis. We checked on our 52 signature positions against these new genomes and found only 2 of them that showed an entropy value slightly over our threshold –0.4. These are PB1-F2 87 and HA 237, with entropy values of –0.522, and –0.692, respectively. The choice of entropy threshold would also affect the number of signatures found. Originally we chose –0.4 on the basis of the value –0.379, computed from PB2 627 by using 95 avian genomes. We noticed that this entropy value reduced to –0.299 at PB2 627 (see online Appendix Table 4) at the later validation stage, when we found 197 E and 19 K from a total of 215 avian PB2 sequences. If we chose to use a more stringent entropy threshold of –0.3, our analysis still showed 46 of those 52 reported signatures; missing were positions 73, 79, and 82 from PB1-F2, 409 from PA, and 237 and 389 from HA.

In addition to the data limitations, this approach of looking for species-associated signatures by entropy is less useful for HA and NA genes. The genetic diversity that exists in either human or avian viruses for these 2 gene segments can markedly boost their respective entropy to more negative values, thus making it difficult to find residues conserved enough for identifying such signatures. We additionally performed the analysis on human H1, H2, and H3 versus avian HA (online Appendix Figure 1). For NA we performed the analysis on human N1 and N2 versus avian NA. We compared 10 human H1, 3 human H2, and 293 human H3 with 95 avian HA sequences and found 13, 13, and 69 signatures (with entropy values for both human and avian within –0.4), respectively. This finding indicates that the human H1 and H2 strains are less distinct from avian strains (H5 dominant) than H3. For NA we found only 6 signatures, in comparison with 8 human N1 versus 95 avian (N1-dominant), and we found only 5 signatures when we compared 298 human N2 and 95 avian sequences. Entropy plots for these analyses can be seen in online Appendix Figure 1.

Two genetic alleles (allele A and B) have been described for the NS gene in avian influenza A virus. We decomposed those 95 avian NS genes into 43 in allele A and 52 in allele B and compared their amino acid sequences with 306 human NS genes. For NS1, 6 signatures were found between human viruses and avian allele A viruses, and 35 signatures were found between human viruses and avian allele B viruses. For NS2, 3 signatures were found between human viruses and allele A viruses, and 6 signatures were found between human viruses and allele B viruses. These results suggest that avian allele B viruses are more distinct from human viruses than are allele A viruses. Entropy plots and histograms for these analyses can be seen in online Appendix Figure 1 and online Appendix Figure 3 (http://www.cdc.gov/ncidod/EID/vol12no09/06-0276_appG3.htm).

From the histograms, we found that some of the 11 genes vary greatly between human and avian viruses, while some others vary little. No boundaries were found between homopairs and heteropairs for HA, NA, and PB1 for human versus avian viruses. This finding seems reasonable because the 2 recent pandemic strains, the 1957 H2N2 and the 1968 H3N2, both originated from reassortment with avian influenza viruses (HA, NA, and PB1 gene segments were from avian influenza). On the other hand, because histograms of NP, followed by PA and PB2, may be used to distinguish human influenza viruses from avian influenza viruses, perhaps some biologic constraints against the occurrence of reassortment exist for these 3 genes. Both the M and NS genes are less differentiable between these 2 types of influenza A viruses.

NP not only displays a clear boundary between human and avian viruses from histogram analysis but also contains more species-associated amino acid signatures (15 of 52) than other ORFs. In addition to NP, polymerase proteins PB2, PB1, and PA also contain abundant species-associated signatures. Most signatures in these viral RNPs are located on the functional domains related to RNP-RNP interactions that are necessary to form replicase/

transcriptase complex (3P and NP), which suggests that specific combinations of polymerase complex and NP would allow an influenza virus to replicate itself efficiently (Table 1). In addition to RNA-interacting domains, many species-associated amino acid signatures of 3P and NP are located in regions related to nuclear localization signals. Influenza viral replication is highly dependent on nuclear function (*35*), making it worthwhile to further examine the roles of those amino acid signatures on nuclear localization of viral RNP in avian versus human cells. We also noticed that several amino acid signatures in NP are located in the regions that interact with cellular proteins, such as splicing factor (BAT1/UAP56) or MxA, which plays a certain role in cellular antiviral mechanisms. What species-specific host factors may affect influenza viral replication rates is not clear. Biologic experiments are required for further understanding the roles of those amino acid residues and related functional domains in the mechanism of interspecies infection.

PB1-F2 is a novel influenza viral protein translated from alternative initiation of PB1 gene. PB1-F2 of PR8 (H1N1) has been shown to target mitochondria and then trigger host cell apoptosis (*36*). Our previous research has found that several strains contain truncated PB1-F2 (*37*). In this study, 379 of 401 PB1 sequences (in the primary dataset) contained PB1-F2 ≥87 and ≤90 aa. For the other 22 sequences, 2 H3N2 strains missed a start codon, 3 H3N2 had the translation stopped at 11 aa, 1 H9N2 stopped at 8 aa, 5 H1N1 stopped at 57 aa, and 3 H9N2 and 7 H3N2 stopped at 79 aa. One H5N1 contained extra residues; its PB1-F2 was 101 aa. We also noted 5 species-associated signatures on PB1-F2; all of them are within the C-terminal domain, which is important for mitochondria targeting (*15,16*). Further investigation of the mitochondria localization of those PB1-F2 variants and their abilities for triggering apoptosis in cells derived from different species is warranted.

How many mutations would make an avian virus capable of infecting humans efficiently, or how many mutations would render an influenza virus a pandemic strain, is difficult to predict. We have examined sequences from the 1918 strain, which is the only pandemic influenza virus that could be entirely derived from avian strains. Of the 52 species-associated positions, 16 have residues typical for human strains; the others remained as avian signatures. The result supports the hypothesis that the 1918 pandemic virus is more closely related to the avian influenza A virus than are other human influenza viruses (*2*). From the 21 avian viruses isolated from humans in this study, we found 19 (90.5%) that contain ≥1 change at the species-associated sites. Upon examining signature changes from similarly sized sets of randomly selected human viruses, randomly selected avian viruses, and randomly selected viruses (avian plus human), we found 29.4%, 71.4%, and 47.1%, respectively, contain species-associated mutations. Although predicting the emergence of a pandemic strain is difficult, close monitoring of how those species-associated signature positions have changed from bird-specific to human-specific signatures may provide a measurement for the prediction of such events.

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Changing Pattern of Human Listeriosis, England and Wales, 2001–2004

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Microbiologic and epidemiologic data on 1,933 cases of human listeriosis reported in England and Wales from 1990 to 2004 were reviewed. A substantial increase in incidence occurred from 2001 to 2004. Ten clusters (60 cases), likely to represent common-source outbreaks, were detected. However, these clusters did not account for the upsurge in incidence, which occurred sporadically, predominantly in patients ≥60 years of age with bacteremia and which was independent of sex; regional, seasonal, ethnic, or socioeconomic differences; underlying conditions; or *Listeria monocytogenes* subtype. The reasons for the increase are not known, but since multiple *L. monocytogenes* strains were responsible, this upsurge is unlikely to be due to a common-source outbreak. In the absence of risk factors for listeriosis in this emerging at-risk sector of the population, dietary advice on avoiding high-risk foods should be provided routinely to the elderly and immunocompromised, not just to pregnant women.

The bacterium *Listeria monocytogenes* and the disease listeriosis were first recognized in laboratory animals in 1924 (*1*). The disease also affects humans, most commonly the unborn, neonates, the immunocompromised, and the elderly. Listeriosis manifests primarily as abortion, septicemia, or central nervous system (CNS) infections, with a high case-fatality rate in all patient groups. Although most cases are foodborne, the epidemiology is complex (*2*). The ubiquitous nature of the bacterium, together with a varied incubation period (1 to >90 days), means that identifying specific food vehicles can be problematic (*3*).

Human listeriosis was very rare in England and Wales during the 1960s and 1970s but increased at the end of the 1980s. From 1987 to 1989, the incidence doubled, probably due to the consumption of contaminated pâté. After a specific brand of pâté was withdrawn from retail sale and warnings were issued to pregnant women and the immunocompromised to avoid eating this food, the incidence declined (*4*). Throughout the 1990s, the average annual total of cases was 110, but the numbers increased to 146, 136, 237, and 213 cases in 2001, 2002, 2003, and 2004 respectively (*5*). We describe preliminary surveillance data on human listeriosis in England and Wales from 1990 to 2004 and speculate on reasons for the upsurge.

Materials and Methods

The Health Protection Agency Centre for Infections (CFI) coordinates routine surveillance of human listeriosis in England and Wales. Case ascertainment is by the voluntary reporting of laboratory-diagnosed cases from microbiology laboratories through an electronic reporting system, or by referral of cultures for identification and subtyping. Epidemiologic and microbiologic data from both systems are combined, checked for duplication, and stored in a database. Additional demographic and clinical data are sought from the responsible consultant medical microbiologists and local health protection teams with a standard questionnaire.

L. monocytogenes isolates from patients with clinical cases, food, and the environment referred to CFI are confirmed phenotypically (*6*) or by PCR (*7*). Isolates are characterized by serotyping (*8*,*9*), phage typing until 2003 (*10*), amplified fragment-length polymorphism (AFLP) analysis since 2002 (*11*), and pulsed-field gel electrophoresis since 2003 on selected isolates (*12*).

For surveillance purposes, a patient with listeriosis was defined as one with a compatible illness from whom *L. monocytogenes* was isolated from a normally sterile site,

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usually blood or cerebrospinal fluid (CSF). Cases were categorized as pregnancy associated (all maternal-fetal patients and neonatal patients; a mother-baby pair was considered 1 case-patient) and nonpregnancy associated (in a patient ≥1 month of age). Case-patients were categorized further into those with CNS infections (*L. monocytogenes* isolated from CSF or brain tissue, clinical evidence of infection of this organ, or both); bacteremia in the absence of CNS infections (*L. monocytogenes* isolated from blood but not from CNS and without clinical evidence of CNS infection); and other conditions not included in the previous 2 categories.

Data manipulation was undertaken in Microsoft Access 2003 (Microsoft Corporation, Redmond, WA, USA) and MapInfo version 8.0 (MapInfo Corporation, Troy, NY, USA). Ethnicity (categorized as ethnic or nonethnic) was assigned by using patients' names (surname and first name when available); patients' ages were grouped into 10-year bands. Indexes of deprivation for England (2004 [*13*]) and Wales (2005 [*14*]), ranked and arranged into quintiles (1 = most deprived and 5 = least deprived areas) and linked to patients postal codes, were used as an approximate marker for patients' socioeconomic status. Patients' postal codes were also used as a marker for patients' residency. Internet searches were used to determine if residential care homes were situated in that postal code area or whether the housing was purely residential.

Data analysis was performed with Microsoft Excel, EpiInfo version 6.04b (Centers for Disease Control and Prevention, Atlanta, GA, USA) and Stata version 8.2 (StataCorp, College Station, TX, USA). Age-specific denominator data from 1990 to 2004 were obtained from the Office for National Statistics. Relative proportions and changes in relative proportions with time were compared by using the χ^2 test and the χ^2 test for trend, respectively. Point estimates of relative risks (RRs) with accompanying 95% confidence intervals (CIs) and significance tests were also calculated.

Results

All Reported Cases

From January 1, 1990, to December 31, 2004, a total of 1,933 reported cases of human listeriosis in England and Wales fulfilled the case definition. Of these 1,377 (71%) were reported through the electronic surveillance system, 1,592 (82%) by isolate referral and 1,068 (55%) by both means. During the study period, the proportion of isolates referred did not change (χ^2 for trend p = 0.94); the proportion of electronic reports received increased slightly (χ^2 for trend p = 0.04). A total of 1,776 patients were admitted to the hospital. For 1,187 patients for whom outcome data were available, 522 (44%) died. From 1990 to 2000, the mean annual incidence was 2.13 cases/million/year (95% CI 2.01–2.25), which increased significantly to 3.47 cases/million/year (95% CI 3.22–3.73) from 2001 to 2004 (RR 1.39, 95% CI 1.31–1.47, p<0.001).

Clustered Cases

Epidemiologic and subtyping analysis identified 10 clusters of cases, which affected 60 patients and likely reflected common-source outbreaks (*15*,*16*); these are summarized in Table 1. When these cases were excluded, a significant increase in disease in 2001–2004 compared with 1990–2000 remained (RR 1.34, 95% CI 1.26–1.42, p<0.001). Subsequent analysis is confined to 1,873 sporadic cases unless otherwise indicated.

Trends in Sporadic Cases

In 1990, sporadic nonpregnancy-associated listeriosis accounted for 80% of the 114 cases reported, and in 2004 for 90% of the 205 cases reported (χ^2 for trend p<0.001; Figure 1). A total of 510 (44%) of 1,155 of the nonpregnancy-associated patients and 29 (10%) of 287 of the sporadic pregnancy-associated patients died.

Data on patients' age were available for 1,543 (97%) of the 1,586 nonpregnancy-associated cases. In 2001–2004, the risk for nonpregnancy-associated listeriosis in persons ≥60 years of age increased by almost half (RR 1.49, 95% CI 1.39–1.60, p<0.001) compared with 1990–2000 (Figure 2). After 2000, the risk among 70- to 79-year-olds (RR 1.32, 95% CI 1.20–1.45, p<0.001) and ≥80-year-olds (1.51, 95% CI 1.33–1.71, p<0.001) was significantly higher than for 60- to 69-year-old patients. Sporadic patients ≥60 years of age were more likely to die (405 [49%] of 828) than those <60 years of age (97 [31%] of 315, χ^2 p<0.001), and the likelihood of death increased with increasing age in this group (χ^2 for trend, p = 0.01).

Data on sex were available for 1,542 (99%) of the 1,543 nonpregnancy-associated patients for whom age was also available. The increased risk in persons ≥60 years of age during 2001–2004 compared with the risk in 1990–2000 was observed in both men (RR 1.47, 95% CI 1.34–1.62, p<0.001) and women (RR 1.49, 95% CI 1.34–1.66, p<0.001) and occurred in most regions of England and in Wales (Figure 3).

Specimen collection dates were available for 1,088 (99%) of 1,102 nonpregnancy-associated patients ≥60 years of age; 503 (46%) cases occurred from July to October. This pattern did not differ for nonpregnancy-associated patients ≥60 years of age reported from 2001 to 2004 (206 [47%] of 442) compared with those reported from 1990 to 2000 (297 [46%] of 646, χ^2 p = 0.84).

Serotyping data were available for 889 (81%) of the 1,102 nonpregnancy-associated patients ≥60 years of age. Serotypes 4b (436 [49%]) and 1/2 (430 [48%]) accounted

Table 1. Clusters of human listeriosis, England and Wales, 1993–2004*

Year	Area	No. cases	Pregnancy associated	*Listeria monocytogenes* type				Vehicles of infection
				Serovar	AFLP	Phage	PFGE	
Clusters probably or likely to be common-source foodborne outbreaks								
1999a	NE England	4	0	4b	ND	ND	ND	Hospital sandwiches
2003	NE England	17	11	4b	V	A	2	Butter
2003	NE England	18	0	4b	I	G	1	None identified
2003	S Wales	2	0	1/2a	XI	Y	L	Hospital sandwiches
2003b	SW England	5	5	1/2a	III	Y	A	Hospital sandwiches
2004	E Midlands	6	0	4b	I	ND	E	None identified
				4b	IV	ND	M	None identified
				4b	V	ND	J	None identified
2004	SE England	2	0	4b	I	ND	A	Hospital sandwiches
				4b	V	ND	B	Not identified
Episodes of neonatal cross-infection								
1990	SE England	2	2	4b	ND	ND	ND	Contact between patients within a delivery suite
1997	SE England	2	2	4b	ND	H	ND	Contact between patients within a delivery suite
1998	SE England	2	2	1/2a	ND	I	ND	Contact between patients within a delivery suite

*AFLP, amplified fragment-length polymorphism; PFGE, pulsed-field gel electrophoresis; NE, northeast; ND, not done; NT, nontypable; SW, southwest; SE, southeast. Two clusters have previously been described: a (*15*) and b (*16*).

for most cases from 1990 to 2004; this proportion did not change significantly during the study period (χ^2 for trend, p = 0.13). No significant differences were observed between patients infected with serotypes 1/2 or 4b in terms of age distribution or death. AFLP typing, applied to cultures collected from 2002 to 2004 and used in conjunction with serotyping, was available for 267 (99%) of 269 nonpregnancy-associated patients ≥60 years of age. Fourteen, 18, and 16 different subtypes were reported in 2002, 2003, and 2004 respectively; 2, 4, and 4 subtypes, respectively, were unique to these years. Ten subtypes were responsible for 241 (90%) of cases, and no individual type occurred more frequently than 22% in any individual year or among all cases.

Data on underlying illness were available for 830 (75%) of 1,102 nonpregnancy-associated patients ≥60 years of age; this proportion did not differ during 1990–2000 (90%) and 2001–2004 (89%). A single underlying condition was reported for 635 (77%) patients, >1 (12%) underlying condition was reported for 97 patients, the specific underlying condition was not recorded for 10 patients (1%), and 88 patients had no underlying condition. No significant change in the underlying conditions reported for patients occurred in 1990–2000 compared with 2001–2004 (Table 2).

Among all nonpregnancy-associated patients, 380 (24%) had evidence of CNS infections, 1,114 (70%) had bacteremia without CNS infections, 59 (4%) had other conditions, and 33 (2%) could not be categorized. The proportion of nonpregnancy-associated patients ≥60 years of age with bacteremia alone increased significantly from 2001–2004 compared with 1990–2000 (85% vs. 76%, χ^2 p = 0.0004) (Figure 4). This difference in proportion was not observed in nonpregnancy-associated patients <60 years of age (65% vs. 59%, χ^2 p = 0.3). Among nonpregnancy-associated patients ≥60 years of age with CNS involvement, the proportion with an accompanying blood-culture isolate from 2001 to 2004 (44 [73%] of 60) was not significantly different than the rate from 1990 to 2000 (98 [65%] of 151, χ^2 p = 0.24). Among all 264 pregnancy-associated patients, the proportion with a blood-culture isolate from 2001 to 2004 (40 [63%] of 64) was not significantly different from that in 1990–2000 (101 [51%] of 200, χ^2 p = 0.09).

Patients' names were available for 1,092 (99%) of 1,102 nonpregnancy-associated patients ≥60 years of age; these were used as a marker for ethnicity. Most patients (1,040, 95%) were classified as nonethnic on the basis of their name. This proportion did not change from 1990–2000 (623 [96%] of 650) to 2001–2004 (417 [94%] of 442, χ^2 p = 0.44).

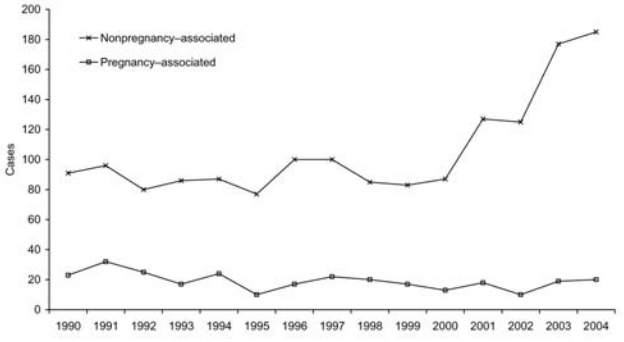

Figure 1. Sporadic cases of listeriosis reported in England and Wales, 1990–2004.

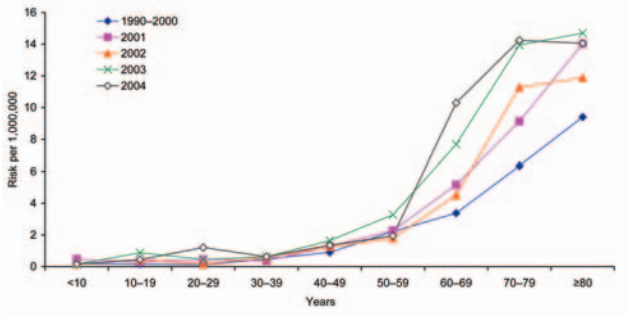

Figure 2. Risk for sporadic nonpregnancy-associated listeriosis by age group, England and Wales, 1993–2004. Individual data shown for years 2001–2004.

Patients' postal codes were available for 634 (58%) of 1,102 nonpregnancy-associated patients ≥60 years, and indexes of deprivation were determined for 563 (89%). The proportion of patients who fell into the quintiles of deprivation 1–5 did not change during the surveillance period (χ^2 for trend p = 0.57, 0.69, 0.64, 0.05, and 0.14, respectively).

Internet searches of the areas covered by the postal codes of the 634 nonpregnancy-associated patients ≥60 years of age showed that, when genuine postal codes were supplied (628, 99%), most (580, 92%) did not contain a residential care home. This proportion did not differ from 2001–2004 (27 [9%] of 309) when compared with 1990–2000 (21 [7%] of 319, χ^2 p = 0.31).

Discussion

Routine surveillance of human listeriosis in England and Wales showed an upsurge in cases such that the annual incidence is now comparable with other European countries with higher incidence (*17*). The clinical manifestations have also changed: bacteremia in older patients without CNS involvement predominates. Several confounding factors could explain the increase in cases and changes in signs and symptoms.

Changes in reporting or referral could have accounted for the observed increase in incidence. The surveillance of listeriosis in England and Wales is passive, and such systems are prone to both underascertainment and pseudo-outbreaks following increased interest in the public health community. Although reporting artifacts cannot be excluded, we are unaware of increased interest in listeriosis from 2001 onwards. Furthermore, reporting and referrals did not change enough to explain the increase.

Improvements in laboratory methods (especially in the isolation of *L. monocytogenes* from blood) or changes in local clinical practice (e.g., more detailed investigations of patients with acute febrile illness seeking primary care) might explain the increase in cases diagnosed or the altered clinical manifestations. We are unaware of substantial changes in blood culture techniques used in England and Wales in the past decade that would increase the diagnosis of listeriosis. Furthermore, although the introduction of mandatory reporting of methicillin-resistant *Staphylococcus aureus* bacteremia in England in 2001 has led to an increase in blood cultures being taken, this is insufficient to explain the increase or shift in clinical manifestations described here (*18*,*19*). Further evidence that the increase was not due to improved diagnostics is the absence of statistically significant increase in the isolation of *L. monocytogenes* from blood cultures from patients with CNS infections or from pregnancy-associated patients.

Demographic changes in the population might have resulted in an overrepresentation of patients from particular age groups without a true increase in risk. Life expectancy in the United Kingdom is increasing; therefore, an increase in listeriosis in older patients is likely to occur. However, calculations controlling for the changing age structure in England and Wales during the surveillance period generates a consistent increase in risk among those ≥60 years of age. Medical advances have resulted in the UK population's surviving for longer with chronic conditions (*20*) with a likely increased susceptibility to listeriosis. While the denominator data required to examine such changes in detail are unavailable, changes would be unlikely to result in an almost 3-fold increase in a single patient age group in a short period without a concomitant increase in younger patients with similar underlying conditions.

Changes in the pathogenicity of *L. monocytogenes* might explain the change in disease manifestations. However, the increase has been due to multiple subtypes, which makes this unlikely. Furthermore, since the upsurge was confined to a restricted patient age group, it is more likely to reflect increased incidence through higher exposure that accompanies behavioral changes.

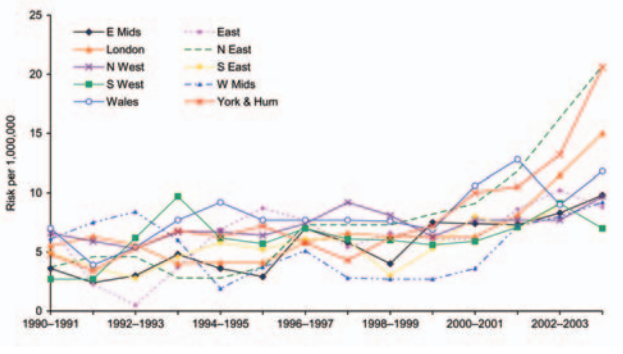

Figure 3. Biannual risk for sporadic nonpregnancy-associated listeriosis in patients ≥60 years of age, by region, England and Wales, 1990–2004. E Mids, East Midlands; East, East of England; N East, Northeast England; N West, Northwest England; S East, Southeast England; S West, Southwest England; W Mids, West Midlands, Wales; York & Hum, Yorkshire and the Humber.

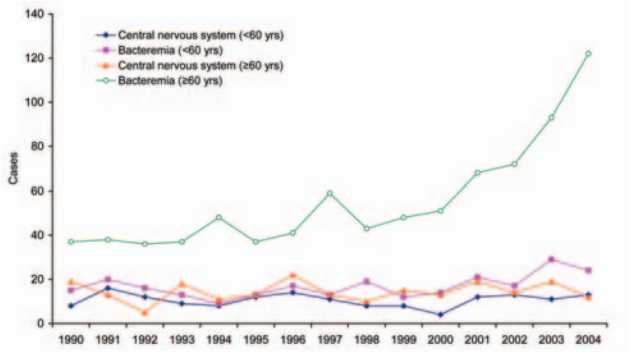

Figure 4. Sporadic nonpregnancy-associated listeriosis in patients with central nervous system infections and bacteremia alone, England and Wales, 1990–2004.

Table 2. Underlying conditions reported for sporadic nonpregnancy-associated listeriosis patients ≥60 years of age, England and Wales, 1993–2004

Classification	1993–2000, n (%)	2001–2004, n (%)	Total
Cancers	173 (42)	143 (43)	316
Autoimmune disorders	53 (13)	46 (14)	99
Cardiovascular disorders	54 (13)	39 (12)	93
Alcohol-related disorders	14 (3)	12 (4)	26
Renal disorders	12 (3)	16 (5)	28
Diabetes	11 (3)	10 (3)	21
Hepatic and biliary disorders	9 (2)	4 (1)	13
Immunosuppressed	1 (0)	4 (1)	5
Postoperative	2 (0)	3 (1)	5
Multiple pathologic conditions	59 (14)	38 (12)	97
Other pathologic conditions	19 (5)	10 (3)	29
Not specified	6 (1)	4 (1)	10
Total	413	329	742

Having examined the most plausible sources of bias, we believe that the observed upsurge and altered clinical manifestations are genuine. Indeed, historical data suggest that the current picture merely represents a continued shift in the epidemiology and clinical manifestations of *L. monocytogenes* infection in England and Wales (Table 3 (*4*,*21*).

The routine epidemiologic and microbiologic data collected for cases of listeriosis in England and Wales are not exhaustive; therefore, our retrospective examination of the factors that have contributed to this upsurge is preliminary. Nevertheless, we have demonstrated that the upsurge is independent of sex; regional, seasonal, ethnic, or socioeconomic differences; underlying conditions; or *L. monocytogenes* subtypes. Furthermore, most older patients in the surveillance period did not reside in care homes and were therefore unlikely to have changed exposure to institutional catering in such settings. UK food consumption/expenditure data also suggest that no major shift in the consumption of major food groups by the older population has occurred in recent years to explain the increase (*22*).

Investigations are continuing to establish the causes of the increase and include application of discriminatory subtyping of *L. monocytogenes* isolates, coupled with the collection of standardized clinical and epidemiologic data for all patients. Hopefully, such steps will facilitate outbreak detection and help identify their cause, as well as enable investigations of factors specific to *L. monocytogenes* subtypes among sporadic cases. However, analytical epidemiology (including case-control studies) and molecular fingerprinting of isolates have not always successfully identified the appropriate interventions to control outbreak-associated and sporadic listeriosis, which suggests that new approaches to investigation are required. Therefore, in the absence of risk factors for listeriosis in this emerging at-risk sector of the population, dietary advice on the avoidance of high-risk foods should be provided routinely to the elderly and immunocompromised and not just pregnant women.

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Table 3. Risk perspective for listeriosis in England and Wales, 1967–2004

Years	Cases per year	Percentage		Outbreaks	Reference
		Pregnancy associated	<60 y of age (bacteremia)		
1967–1985	<75–136	33	31 (16)	Some clusters (foodborne?)	(*4*)
1987–1989	237–278	40	–	50% in 1 outbreak (pâté)	(*21*)
1990–2000	87–128	19	67 (49)	Limited clusters	This study
2001–2004	136–237	11	73 (58)	Largely sporadic, some clusters	This study

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Predominance of Ancestral Lineages of *Mycobacterium tuberculosis* in India

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Although India has the highest prevalence of tuberculosis (TB) worldwide, the genetic diversity of *Mycobacterium tuberculosis* in India is largely unknown. A collection of 91 isolates originating from 12 different regions spread across the country were analyzed by genotyping using 21 loci with variable-number tandem repeats (VNTRs), by spoligotyping, by principal genetic grouping (PGG), and by deletion analysis of *M. tuberculosis*–specific deletion region 1. The isolates showed highly diverse VNTR genotypes. Nevertheless, highly congruent groupings identified by using the 4 independent sets of markers permitted a clear definition of 3 prevalent PGG1 lineages, which corresponded to the "ancestral" East African–Indian, the Delhi, and the Beijing/W genogroups. A few isolates from PGG2 lineages and a single representative of the presumably most recent PGG3 were identified. These observations suggest a predominance of ancestral *M. tuberculosis* genotypes in the Indian subcontinent, which supports the hypothesis that India is an ancient endemic focus of TB.

Tuberculosis (TB) in humans has been described since ancient times. *Mycobacterium tuberculosis*, its main causative agent, is widely disseminated and is one of the most successful human pathogens today, with 2 billion persons infected. Most of the disease's effects are now concentrated in countries with few resources; India has the highest number of cases (*1*).

Because of the clonal structure of *M. tuberculosis* (*2–4*), comparative genotypic analyses from widespread geographic areas, such as the Indian subcontinent, or from different human populations can give unique insights into dissemination dynamics and evolutionary genetics of the pathogen (*5*,*6*). IS*6110* restriction fragment length polymorphism–based fingerprinting (*7*) has been used to study the mycobacterial population structure from southern India, northern India, and the Delhi region (*8–11*). However, IS*6110* fingerprinting is of limited use because a high proportion of *M. tuberculosis* strains have low copy numbers or are devoid of IS*6110* in several regions of India (*8*,*10*). IS*6110* typing also has a relative lack of portability, which hinders comparison between separate studies (*12*). Fingerprinting methods targeting polymorphic spacer sequences in the direct repeat (DR) region, including spoligotyping, have been used in some of these regions and in Bombay (*13–15*). However, when used alone, these methods considerably underestimate the clonal diversity (*16*). Because of these limitations, knowledge about the mycobacterial population structure in India remains incomplete.

More recently, molecular typing methods based on variable-number tandem repeats (VNTRs) of genetic elements named mycobacterial interspersed repetitive units (MIRUs) (*17*) have been developed (*18*,*19*). MIRU-VNTR typing shows a discriminatory power close to that of IS*6110* fingerprinting and is particularly efficient in distinguishing *M. tuberculosis* isolates with few IS*6110* elements or none (*19–21*). MIRU-VNTRs are sufficiently stable to track epidemic strains (*19*,*20*,*22*).

We analyzed *M. tuberculosis* strain diversity in a sample of 91 isolates from 12 different regions, including northern, central, and southern India, by using a set of 21 VNTR loci, including the 12 MIRU-VNTR loci described previously (*17*,*18*) and 9 additional loci containing VNTRs

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of other interspersed genetic elements (*23*–*25*). All of these loci are collectively designated MIRU-VNTR loci in this study. Spoligotyping was used as a complementary technique because this procedure, albeit less discriminatory, is useful in identifying genotype families (*16*,*26*,*27*). In addition, single nucleotide polymorphism (SNP) genotyping on the *katG* and *gyrA* genes and genomic deletion analysis with *M. tuberculosis*–specific deletion region 1 (TbD1) were used to assess consistency of the genetic relationships obtained by VNTR typing and spoligotyping at a broader evolutionary level. SNPs in the *katG* and *gyrA* genes classify *M. tuberculosis* isolates into 3 principal genetic groups (PGGs) thought to have evolved sequentially from group 1 to group 3 (*2*). TbD1 is specifically present in a subset of PGG1 strains, but absent in other strains of PGG1, and in PGG2 and PGG3 strains; TbD1+ strains have therefore been proposed to constitute an ancestral lineage of *M. tuberculosis* (*28*). Using a combination of all 4 markers, we found that ancestral lineages prevail in our collection, which suggests an ancient focus of TB in the Indian subcontinent.

Materials and Methods

Strains and Genomic DNA Extraction

A sample of 100 clinical isolates of *M. tuberculosis* was initially selected; the isolates originated in 12 different regions, from northern, central, and southern India and part of eastern India (Table 1). For 9 isolates, mixed infections or laboratory cross-contamination was suspected after MIRU-VNTR typing (see Results), and they were excluded from further analysis. The isolates were collected from patients with pulmonary TB who had voluntarily visited their nearest medical college or hospital for diagnosis and treatment. Therefore, in most of the cases, the patients lived near the respective cities reported in Table 1. Patients were adults, 20 to 45 years of age, and represented both men and women, except those from Ranchi where all reported cases were in male army personnel. Information regarding the extent of disease and treatment status (new or recurrent cases of disease) was not available. From these hospitals, most of the isolates (designated hereafter as ICC, VA, VK, HA, BC, and ASN) were transported to the repository collection maintained at the Jalma Institute in Agra for further characterization and drug susceptibility testing, which was performed by the proportion method. Table 2 includes sensitivity profiles of isolates tested at the Jalma laboratory. The isolates from New Delhi were collected between March 1997 and March 1998, from Ahmedabad between November 1996 and July 1997, from Ranchi between February and March 1999, from Chandigarh in September 1997, from Bangalore in November 1996, from Jammu between May and July 2001, from Jaipur in May 2001, from Agra in May 2001, and from Varanasi between June and November 1999. The isolates designated as MHRC were from pulmonary TB patients who sought treatment at the Mahavir Hospital, Hyderabad, between 2000 and 2002. The isolates designated as TRC were from pulmonary TB patients at the Tuberculosis Research Centre, Chennai. *M. tuberculosis* DNA was extracted by using the standardized protocol as described (*7*).

Table 1. Origin of the *Mybacterium tuberculosis* isolates genotyped in this study

Origin	No. isolates*
Agra	2
Ahmedabad	7
Bangalore	6
Chandigarh	9
Chennai	9
Haridwar	2
Hyderabad	10
Jaipur	5
Jammu	6
New Delhi	26
North India	1
Ranchi	5
Shimla	1
Varanasi	2

*Nine isolates, originally selected and for which mixed infection or laboratory cross-contamination was suspected after typing with mycobacterial interspersed repetitive unit–variable-number tandem repeats (see Results), are not included.

TbD1 Analysis

The presence of TbD1 was analyzed by PCR (*28*). Briefly, 2 PCR assays were performed per isolate tested, by using either primers complementary to the sequences flanking the deleted region or primers complementary to the internal sequences. For the isolates that did (TbD1+) or did not (TbD1–) contain the TbD1 region, an amplicon was obtained only with internal primers or only with flanking primers, respectively.

Single Nucleotide Polymorphism Analysis

To define the PGGs, the polymorphisms at the *katG* codon 463 and the *gyrA* codon 95 were determined by sequence analysis after PCR amplification with the same primers as in Sreevatsan et al. (*2*). The amplification products were sequenced by using an ABI 3700 DNA sequencer and the BigDye Terminator v3.1 Cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA).

Spoligotyping

Spoligotyping was performed by using a commercial kit (Isogen Bioscience BV, Maarsen, the Netherlands) according to the previously described method (*29*). Reverse blotting analysis of spacer sequences in the DR

Table 2. Available drug susceptibility profiles to rifampin (Rif) and isoniazid (Inh) of *Mycobacterium tuberculosis* isolates

Isolate no.	Origin	Sensitivity to Rif, Inh*
ICC-101	New Delhi	S, R
ICC-102	New Delhi	S, R
ICC-103	New Delhi	R, R
ICC-104	New Delhi	R, R
ICC-105	New Delhi	R, R
ICC-107	New Delhi	S, S
ICC-109	New Delhi	S, R
ICC-114	New Delhi	S, R
ICC-1141	Jammu	S, S
ICC-124	New Delhi	S, R
ICC-128	New Delhi	R, R
ICC-132	Ahmedabad	S, S
ICC-133	Ahmedabad	R, R
ICC-137	Ahmedabad	S, R
ICC-138	Ahmedabad	S, S
ICC-144	Shimla	S, R
ICC-155	Chandigarh	S, R
ICC-161	Chandigarh	S, S
ICC-173	Chandigarh	S, S
ICC-174	Chandigarh	S, S
ICC-19	New Delhi	R,R
ICC-208	New Delhi	S, S
ICC-211	New Delhi	S, S
ICC-212	New Delhi	S, S
ICC-214	New Delhi	S, R
ICC-217	New Delhi	R, R
ICC-219	New Delhi	S, S
ICC-223	New Delhi	S, S
ICC-23	New Delhi	R, R
ICC-244	New Delhi	R, R
ICC-247	Chandigarh	R, R
ICC-248	Chandigarh	S, S
ICC-251	Chandigarh	S, S
ICC-254	Chandigarh	R, R
ICC-257	Chandigarh	R, S
ICC-275	New Delhi	R, S
ICC-286	New Delhi	R, R
ICC-327	New Delhi	R, R
ICC-33	Ahmedabad	R, R
ICC-332	Jaipur	R, R
ICC-37	Ahmedabad	R, R
ICC-399	Bangalore	R, S
ICC-95	Bangalore	S, S
ICC-96	Bangalore	R, R
ICC-98	New Delhi	S, S

*S, susceptible; R, resistant.

region was performed by using a streptavidin-horseradish peroxidase–enhanced enzyme chemiluminescence assay (Amersham Pharmacia-Biotech, Roosendaal, the Netherlands).

MIRU-VNTR Typing

The *M. tuberculosis* isolates were genotyped by PCR amplification of the 12 MIRU-VNTR loci (*18*) and 9 additional VNTR loci (*23–25*) by using an automated technique as described (*19*). The set of loci thus included (alternative designation in parentheses) MIRU-VNTR loci 2, 4 (ETR-D), 10, 16, 20, 23, 24, 26, 27, 31 (ETR-E), 39 and 40; and VNTR loci 424, 577 (ETR-C), 1895 (QUB-1895), 2347, 2401, 2461 (ETR-B), 3171, 3690, and 4156 (QUB-4156). The primers against the MIRU-VNTR flanking regions were the same as described (*18*), except that hex labeling was replaced by Vic labeling. The primers corresponding to the 9 additional VNTR loci and the conditions for their amplification by multiplex PCR are described in Table 3. The PCR fragments were sized and the various VNTR alleles were assigned after electrophoresis on a 96-well ABI 377 sequencer with customized GeneScan and Genotyper software packages (PE Applied Biosystems), as described (*19*) and on the basis of data described in Table 3. Tables used for MIRU-VNTR allele scoring are available at http://www.ibl.fr/mirus/mirus.html.

Analysis of Genetic Relationships

MIRU-VNTR profiles and spoligotypes were computed as character data into the Bionumerics program (Bionumerics version 2.5, Applied Maths, Saint-Martens-Latem, Belgium). MIRU-VNTR profiles were compared to each other by using the neighbor-joining algorithm. For spoligotypes, the Jaccard index was calculated to allow for the construction of a dendrogram by using the unweighted pair-group method with arithmetic averages. The spoligotypes were compared to fingerprints in an international database (*31*) that contained fingerprints from 13,008 isolates at the time of the consultation (June 2004). The genetic relationships between the isolates based on the MIRU-VNTR types were assessed by matching the spoligotypes with TbD1, and SNP analyses were carried out on a selected set of isolates representative of the different spoligotypes in each of the 3 predicted PGG1 groups and in the predicted PGG2/3 groups.

Results

MIRU-VNTR and Spoligotype Analysis

Nine of the 100 isolates of the study collection displayed 2 alleles in several independent MIRU-VNTR loci among the 21 tested, which suggested mixed DNA populations. These mixed populations could have originated from laboratory cross-contaminations or from mixed infections. Therefore, they were excluded from further analysis.

The remaining 91 isolates showed highly diverse MIRU-VNTR genotypes (Figure 1). Seventy-eight distinct genotypes were detected in this collection, including 6 cluster patterns and 72 unique patterns. The largest MIRU-VNTR cluster included 8 isolates, 5 of which originated in Ranchi. Another cluster included 3 isolates from New

Table 3. Conditions for multiplex PCRs of 9 VNTR loci*

Multiplex	Locus	Conventional designation†	VNTR length (bp)	$MgCl_2$ (mmol)	PCR primer pairs (5′ to 3′, with labeling indicated)
Mix E	46	VNTR 2347	57	1.5	GCCAGCCGCCGTGCATAAACCT (Fam) AGCCACCCGGTGTGCCTTGTATGAC
	48	VNTR 2461	57		ATGGCCACCCGATACCGCTTCAGT (Vic) CGACGGGCCATCTTGGATCAGCTAC
	49	VNTR 3171	54		GGTGCGCACCTGCTCCAGATAA (Ned) GGCTCTCATTGCTGGAGGGTTGTAC
Mix F	42	VNTR 0424	51	1.5	CTTGGCCGGCATCAAGCGCATTATT GGCAGCAGAGCCCGGGATTCTTC (Fam)
	43	VNTR 0577	58		CGAGAGTGGCAGTGGCGGTTATCT (Vic) AATGACTTGAACGCGCAAATTGTGA
	44	VNTR 1895	57		GTGAGCAGGCCCAGCAGACT (Ned) CCACGAAATGTTCAAACACCTCAAT
Mix G	47	VNTR 2401	58	3.0	CTTGAAGCCCCGGTCTCATCTGT (Fam ACTTGAACCCCCACGCCCATTAGTA
	52	VNTR 3690	58		CGGTGGAGGCGATGAACGTCTTC (Vic) TAGAGCGGCACGGGGGAAAGCTTAG
	53	VNTR 4156	59		TGACCACGGATTGCTCTAGT GCCGGCGTCCATGTT (Ned)

*VNTR, variable-number tandem repeats.
†VNTR 0577, 2461, 4156, 1895 correspond to ETRC, ETRB, QUB 4156 and 1895, respectively (*23*,*24*). The conditions for PCR amplification were the same as in (*30*).

Delhi, while the remaining 4 clusters contained 2 isolates each (1 with 2 isolates from Delhi; the others included isolates from Jammu and Chandigarh, from Hyderabad and Chennai, and from Bangalore and Chandigarh). Information about possible links between patients with clustered isolates was not available. The number of different spoligotypes (36 distinct spoligotypes, including 11 cluster patterns and 25 unique patterns) was lower than that of the MIRU-VNTR types, which was consistent with previous comparisons between spoligotyping and MIRU-VNTR systems based on 12 loci (*19*–*21*). None of the MIRU-VNTR clusters was split by spoligotyping, while of the 11 spoligotype clusters, 9 were split by MIRU-VNTR typing.

The genetic relationships between the isolates based on the MIRU-VNTR types by using the neighbor-joining algorithm are displayed in Figure 1. This dendrogram indicates 3 main genotype groups. The identity of these groups was inferred by comparison with genetically well-characterized isolates from a worldwide collection (*16*), typed by using the same 21 MIRU-VNTR loci (*19*; P. Supply et al., unpub. data). These groups are thereby predicted to correspond to the TbD1+ ancestral lineage (41 isolates, 45% of the total isolates), the recently described Delhi or Central Asian (CAS) genogroup (24 isolates, 26%), and the Beijing genogroup (9 isolates, 10%), respectively. These 3 groups belong to PGG1. The remaining isolates (17

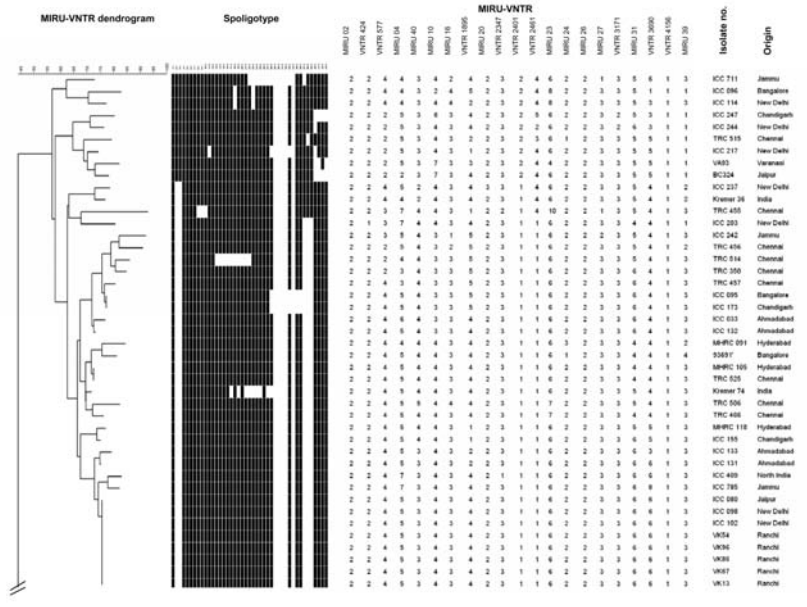

Figure 1. Genetic relationships of Indian *Mycobacterium tuberculosis* isolates. The dendrogram based on mycobacterial interspersed repetitive units–variable-number tandem repeats (MIRU-VNTR) genotypes, generated by using the neighbor-joining algorithm, was rooted with *M. canettii*, the most divergent member of the *M. tuberculosis* complex. Corresponding spoligotypes of the TbD1+/EAI isolates are shown; additional genogroups, principal genetic group (PGG), and *M. tuberculosis*–specific deletion region 1 (TbD1) status are indicated in an expanded version of the figure, available online at http://www.cdc.gov/ncidod/EID/vol12no09/05-0017-G1.html. Indian isolates included in the collection of Kremer et al. (*16*) were included as references.

isolates, 19%) are predicted to belong to PGG2 or PGG3 genogroups.

Congruence of Groupings Between Markers

In accordance with the MIRU-VNTR typing results and the absence or presence of DR spacers 33–36 (*26*), *katG* and *gyrA* sequence analyses identified all tested representatives from Delhi and the ancestral genogroups as PGG1, whereas 14 tested isolates were assigned to PGG2 (Figure 1). One representative of PGG3, assumed to be the most recent group, was detected in this sample. The Beijing/W isolates were not tested for *katG* and *gyrA* polymorphism, since the fact that they all belong to PGG1 is well documented (*26*).

In agreement with Brosch et al. (*28*), we found the TbD1 region in all tested isolates from the predicted ancestral group but not in all tested Beijing/W, PGG2, and PGG3 isolates (Figure 1). We also found that all the tested Delhi isolates lacked TbD1.

Already known spoligotype signatures (*16*,*26–28*, *31*,*32*), and a few new variants, were found within the 4 groups defined by MIRU-VNTR analysis (Figure 1 and Table 4). The TbD1+ isolates were characterized by the absence of spacers 29 to 32 and 34, and (except in 4 cases) by the presence of spacer 33. Most isolates (35 of 43, taking into account 2 Indian isolates from the collection of Kremer et al. [*16*]) also lacked spacers 2 and 3. Based on these results, three fourths of the TbD1+ isolates were included in the spoligotype EAI3 class (*33*), while 1 isolate belonged to the EAI1 class. The remaining TbD1+ isolates represented new EAI variants. EAI classes 2, 4, and 5 were not found in this collection. Typically, the Beijing/W isolates only harbored spacers 35–43 (with spacers 39 and 40 missing in 1 case) (*5*,*32*). As described recently (*11*), the Delhi isolates shared the block of 9 final spacers (with some internal variation) with the Beijing/W strains but included 2 additional blocks among spacers 1–22. They specifically lacked spacers 4–7, and 23–34. The Delhi types are thus included in the CAS spoligotype family (*33*). Fourteen and 4 isolates out of 25 (taking into account 1 Indian isolate from the collection of Kremer et al. [*16*]) conformed to the 2 main spoligotype prototypes, CAS1 and CAS2, respectively (*33*).

As expected, the prototypes of the Latin American–Mediterranean (LAM, 3 cases), X (1 case), and T (8 cases) spoligotype families were detected among the isolates of the PGG2/3group. The single PGG3 isolate (ICC399) had a T spoligotype, which includes both PGG2 and PGG3 strains (*26*,*33*). Highly similar groupings of the isolates were observed when a dendrogram was built based on spoligotypes alone or on a combination of VNTR and spoligotypes, although the resolution was lower when spoligotyping was used alone (data not shown).

Table 4. Specific spoligotype signatures observed in this study*

PGG	TbD1	Class	Spacers in DR region	
			Absent	Present
1	+	EAI1	29–32, 34, 40	All others
1	+	EAI3	2–3, 29–32, 34, 37–39	All others
1	+	EAIx	2–3, 29–32, 34	Most others
1	–	CAS1	4–7, 23–34	All others
1	–	CAS2	4–10, 23–34	All others
1	–	CASx	4–7 or 4–10, 23–34	Most others
1	–	Beijing	1–34	Most others
2–3	–	LAM, X, T	33–36	Most others

*DR, direct repeat; PGG, principal genetic grouping; TbD1, *M. tuberculosis*–specific deletion region 1; EAI, East African–Indian; CAS, Central Asian; LAM, Latin American–Mediterranean.

Comparison of Spoligotypes with an International Database

Table 5 shows the results from a comparison of the Indian spoligotypes with SpolDB3.0, a database containing data from >13,000 *M. tuberculosis* complex isolates obtained worldwide (*31*). Of 36 different spoligotypes found in the Indian strains, 15 (41.7%) were not present in SpolDB3.0. Most (11, 73%) of these new spoligotypes correspond to PGG1 isolates. Conversely, only 1 Indian isolate had the second most frequent spoligotype worldwide, S53. These observations reflect the current underrepresentation of strains from India in SpolDB3.0 (n = 44).

Discussion

This report describes the diversity of *M. tuberculosis* strains obtained from patients in various regions in India, relying on a conveniently available set of isolates collected

Table 5. Major spoligotypes in India*†

Spoligotype	India, no. (%)	SpolDB3.0, no. (%)	Geographic distribution‡
S11§	27 (28.7)	121 (1.03)	ASI, EUR, OCE, NAM, CAM, SAM
S26§	14 (14.9)	102 (0,87)	ASI, EUR, OCE, NAM, CAM
S1§	8 (8.5)	1,282 (10.95)	Ubiquitous
S52§	4 (4.3)	163 (1.39)	Ubiquitous
S288§	4 (4.3)	6 (0.051)	ASI, EUR, OCE, NAM
S138§	2 (2.1)	23 (0.20)	EUR, NAM, CAM
S342§	2 (2.1)	3 (0.026)	NAM
S357§	2 (2.1)	8 (0.068)	ASI, EUR, NAM
S361§	2 (2.1)	3 (0.026)	ASI, EUR
ICC09¶	2 (2.1)	–	India
ICC114¶	2 (2.1)	–	India

*n = 94.
†Three Indian isolates from the collection of Kremer et al. (*16*) were included as references. Only spoligotypes shared by ≥2 isolates are shown.
‡Ubiquitous, spoligotype present in all 7 geographic areas; AFR, Africa; ASI, Asia; EUR, Europe; OCE, Oceania; SAM, South America; CAM, Central America; NAM, North America.
§Designation of the spoligotype in SpolDB3.0.
¶New spoligotype not included in SpolDB3.0.

between 1997 and 2002. While these data are not representative of all TB patients in those regions and lack information regarding clinical characteristics, they provide valuable first insights into the diversity of circulating *M. tuberculosis* strains in this country. The excellent congruence observed between the 4 independent sets of genetic markers used here lends strong support to the assignment of different prevalent lineages. This congruence is consistent with the clonal population structure of *M. tuberculosis* (*2–4*) and reflects the respective informative values of the markers used. In particular, the results show that the use of a large set of VNTR loci simultaneously allows for both reliable identification of genogroups and high-resolution analysis of intralineage diversity, without the limitations that apply to IS*6110* fingerprinting or other typing methods used in the few previous molecular studies on Indian isolates. Within the framework of the current evolutionary scenario of *M. tuberculosis*, which proposes phylogenies based on PGGs and genomic deletion analyses (e.g., TbD1) (*2*,*3*,*28*), we found a striking prevalence of ancestral genotypes (TbD1+) and the concurrent poor representation of the most recent lineages in this Indian collection (PGG2 and especially PGG3). This finding contrasts with the situation in other regions of the world, such as Europe and North and South America, where PGG2 and PGG3 constitute most of the *M. tuberculosis* strains (*31*).

Ancestral isolates of *M. tuberculosis* are characterized by the presence of the TbD1 region, which has been recently identified as an evolutionary landmark in the genome of this species. This region was detected initially in a few *M. tuberculosis* strains belonging to PGG1, as well as in *M. canettii*, *M. bovis*, *M. africanum*, and *M. microti*, whereas this region was shown to be absent in all PGG2 and PGG3 strains as well as in the other PGG1 strains tested (*28*). The grouping of all the tested TbD1+ isolates by MIRU-VNTR typing and spoligotyping (*16*,*19*, this study) support their assignment to a single lineage (*28*), the East African–Indian lineage (*27*). Consistently, all tested representatives of known modern *M. tuberculosis* genotype families were TbD1–. A similar systematic association has recently been observed in strains from Singapore (*34*) and from Bangladesh (*35*), which supports the notion that the deletion of TbD1 occurred as a single evolutionary event in a common ancestor rather than on independent multiple occasions (*28*).

In this study, all isolates that contain ≥2 repeats in MIRU-VNTR locus 24 belong to the ancestral (TbD1+ group, and all but 2 isolates containing 1 repeat unit in locus 24 belong to the modern (TbD1–) groups. This correlation, also seen in previous studies on isolates from Singapore (*34*) and Bangladesh (*35*), indicates that this locus alone is highly informative in the identification of ancestral and modern *M. tuberculosis* strains.

The few previously identified TbD1+ strains were isolated from patients from East Africa and South Asia. These strains have low copy numbers of IS*6110* (*16*,*28*) and belong to cluster I within PGG1 (*3*). This lineage is distinct from IS*6110* low-copy-number strains in PGG2 (*3*), which was isolated from patients in English-speaking countries (33). IS*6110* low- copy-number strains are prevalent with variable proportions among patients from several countries in Southeast Asia (*36*), and the analysis of the available spoligotype data suggests that most of them belong to the TbD1+/EAI lineage (*26*,*31*,*37*). Frequencies of TbD1+/EAI isolates have recently been reported to range from 25% to 50% in Bangladesh (*35*,*36*) and Singapore (*34*). A frequency of 8% has been reported in a study that only used spoligotyping for genetic characterization of 105 isolates from the Delhi area (*15*). Until now, the highest prevalence of IS*6110* low-copy-number isolates (≈60%) has been observed in southern India (*8*,*9*,*38*). Consistent with these studies, we found that 80% of the samples obtained from the southern regions from India were TbD1+/EAI isolates, although such isolates were found in nearly all regions (Figure 2). Also consistent with our findings, most spoligotypes observed in an ongoing population-based study (>1,200 isolates) in the southern state of Tamil Nadu were of the EAI3 class (S. Narayanan et al., unpub. data), found to be predominant in our collection (Table 4 and Figure 1).

The prevalence of these low-copy-number strains in regions of such high endemicity has raised the question of the true extent of genetic variation beyond their restricted IS*6110* distribution (*9*). The MIRU-VNTR typing results obtained here indicate that the genetic diversity in the TbD1+/EAI lineage goes far beyond the commonly observed restricted spectrum of IS*6110* low copy-number fingerprints and of known spoligotypes (*31*,*36*). For instance, most isolates with identical spoligotype 11 of the predominant EAI3 class in this study were of different MIRU-VNTR types (Figure 1). Moreover, the EAI lineage contains 3 additional spoligo-prototypes (EAI2, 4, and 5) that were absent from the population studied here. In addition, at least 1 group of TbD1+/EAI strains recently isolated in Singapore had high-copy numbers (up to 15) of IS*6110* (*39*). Altogether these observations indicate that the TbD1+ strains constitute a highly diversified lineage, which is consistent with an ancestral phylogenetic position.

In addition to the TbD1+ isolates, 2 other major PGG1 families were well represented in this Indian collection. They were qualified as modern groups by their TbD1– status. The recently identified Delhi type (*11*), classified as the CAS group by spoligotyping (*33*), represented approximately one fourth of the total sample. This genogroup made up 60 (72%) of 83 isolates collected from male patients attending 1 hospital and a clinic of the Delhi

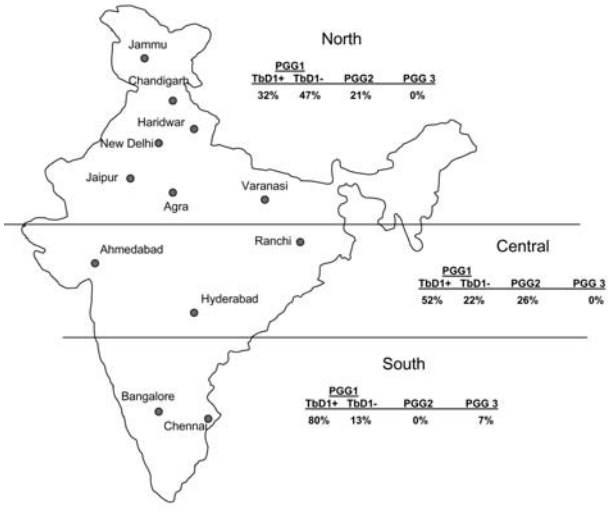

Figure 2. Geographic distribution of *Mycobacterium tuberculosis* genotypes in northern, central, and southern India. PGG, principal genetic group; TbD1, *M. tuberculosis*–specific deletion region 1.

region over a 1-year period (*11*) and 38 (36%) of 105 isolates collected from patients attending other health centers in Delhi (*15*). Although this genogroup is less dominant in this region, representing 5 (20%) of the 26 isolates from Delhi, the Delhi genogroup was well represented among the isolates from northern and central India as well. The second TbD1– PGG1 family detected in this study corresponds to the widespread Beijing/W family, which accounted for 10% of the total sample. Most (7 of 9) of these isolates were from Delhi, where they represented 30% of the isolates studied, in contrast to the 1% to 8% noted in other studies on isolates from Delhi or other Indian regions (*11*,*15*,*32*).

The predominance of *M. tuberculosis* ancestral strains and the relatively poor representation of the most recent lineages in this Indian collection lend support to the hypothesis that India is a relatively ancient endemic focus of TB (*28*). On the basis of these findings, we speculate that the Indian subcontinent was an early step of the worldwide expansion of the *M. tuberculosis* complex, subsequent to the recently proposed emergence of tubercle bacilli in eastern Africa millions of years ago (*40*). However, we acknowledge that, as our collection represents a minuscule fraction of the millions of TB cases in India, genotyping additional isolates from TB patients in this country will be necessary to determine if these initial observations hold true (as suggested by unpublished data from >1,200 isolates from southern Tamil Nadu) or substantially change for a larger fraction of reported cases. Nevertheless, we believe that our data constitute the most solid available foundation for future comparisons of these additional isolates and those obtained from patients in the rest of the world.

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Extrapulmonary Tuberculosis by Nationality, the Netherlands, 1993–2001

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This study describes the epidemiology of extrapulmonary tuberculosis (TB) in the Netherlands from 1993 through 2001. We assessed whether the increasing numbers of inhabitants with a non-Western ethnic background had an effect on the number of extrapulmonary patients. We used data from the Netherlands Tuberculosis Register and included all cases of TB diagnosed in the Netherlands between January 1, 1993, and December 31, 2001. Information on age, sex, nationality, year of diagnosis, culture result, anatomic location of the site of disease, and HIV status was retrieved from the register. Of 13,258 patients with TB, 8,216 (62%) had pulmonary TB, and 5,042 (38%) had extrapulmonary TB. Non-Dutch nationals were more likely to have most types of extrapulmonary TB. The growth of the number of inhabitants with a non-Western ethnic background in the Netherlands explains the proportional growth of extrapulmonary TB. Physicians need to be aware of the changing clinical picture of TB.

Tuberculosis (TB) is a major public health problem, affecting 8 million persons per year worldwide (*1*). The global incidence rate of TB per capita is growing by ≈1.1% per year (*1*). Contrary to the increasing number of TB cases in developing countries, the number of cases in industrialized countries is stable or decreasing (*2–4*). In the United States, a decreasing trend of the total number of TB patients is seen with an increasing proportion of TB cases with extrapulmonary TB, resulting in a rising proportion from 7.8% in 1964 to 20% in 2001 (*5–8*). Both the HIV epidemic and changes in population demographics, with rising numbers of immigrants, are being held responsible for this proportional increase of extrapulmonary TB (*6,7,9*). A recent study of extrapulmonary TB in Hong Kong Special Administrative Region, People's Republic of China showed that 22.3% of the TB cases were extrapulmonary (*10*), while a small Canadian study found a proportion of 46% (*11*). No national studies about extrapulmonary TB in developing countries are known.

Extrapulmonary TB refers to TB outside the lungs. Mycobacteria may spread through lymphatic or hematogenous dissemination to any tract or through coughing and swallowing to the gastrointestinal tract. Bacteria may remain dormant for years at a particular site before causing disease. Since extrapulmonary TB can affect virtually all organs, it has a wide variety of clinical manifestations, which causes difficulty and delay in diagnosis (*7,8*). Obtaining material for culture confirmation of extrapulmonary TB is much more difficult than obtaining material for culture confirmation of pulmonary TB (*7*). Extrapulmonary TB is more often diagnosed in women and young patients (*6,7,9–13*). In the United States, extrapulmonary TB is associated with ethnic minorities and those born in other countries (*6*). In many countries, patients from Asian origin are known to have a higher incidence of extrapulmonary TB, especially lymphatic TB (*11,14–17*). A study of Somali TB patients in Minnesota showed frequent lymphatic TB as well (*18*). In HIV-infected patients the frequency of extrapulmonary TB depends on the degree of decreased cellular immunity (*19,20*). In patients with <100 CD4 cells/mL, extrapulmonary and disseminated TB counts for 70% of all forms of TB (*21*).

The Netherlands has a low incidence of HIV and, similar to other industrialized countries, a decreasing TB incidence rate. TB was a common disease in the nineteenth century, but after the Second World War the incidence rate declined rapidly (*22*). During the last decade, the numbers

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of inhabitants with a non-Western ethnic background has been growing (*23*). The proportion of TB patients with a non-Dutch nationality has increased from 30% in 1980 to 63% in 2000 (*22*,*24*,*25*). Dutch policy regulates the screening by chest radiograph of immigrants from countries with a high prevalence who intend to stay for more than 3 months (*26*). No studies have described the epidemiology of extrapulmonary TB in the Netherlands, with the exception of 1 study on bone and joint TB (*27*).

This article describes the epidemiology of extrapulmonary TB in the Netherlands between 1993 and 2001. Our main focus is on the relation between extrapulmonary TB and nationality. We assess whether the increasing number of inhabitants with a non-Western ethnic background has had an effect on the number of extrapulmonary patients.

Methods

For this study, we used data from the Netherlands Tuberculosis Register (NTR), an anonymous case register held by KNCV Tuberculosis Foundation. Every TB patient reported to 1 of the 45 municipal health services in the Netherlands is included in this register. Reporting to the register is voluntary. Comparison with the mandatory notification system of the Ministry of Health suggests 99% completeness (*28*). We included all cases of TB diagnosed in the Netherlands from January 1, 1993 through December 31, 2001. Information on age, sex, and nationality, as recorded in the passport, year of diagnosis, culture result, anatomic location of the site of disease, and HIV status was retrieved from the register. Non-Dutch nationality was divided into 7 different nationality groups: Europe (central and eastern: Poland, Czech, Slovakia, Hungary, Romania, Bulgaria, Albania and the countries of the former Yugoslavia and the former Soviet Union), Turkey, Morocco, Somalia, Africa (other), Asia, and other countries. HIV testing is not a standard procedure for TB patients in the Netherlands. We considered case-patients with a record of impaired immunity due to HIV infection as HIV infected and all others as HIV status unknown.

Information about the anatomic location of disease was registered using 2 different classification systems. In the NTR, the location of TB is generally divided into 3 categories, pulmonary, extrapulmonary, or combined pulmonary and extrapulmonary disease, and the disease is classified according to the site of the disease by using the International Classification of Diseases 9th revision (ICD-9). We first used the information from the ICD-9 classification to divide the patients into a pulmonary TB group, i.e., major site of disease location inside the lungs, and an extrapulmonary TB group, i.e., the major site of disease was outside the lungs. Thereafter, 8 types of extrapulmonary disease were defined: lymphatic, pleural, bone and/or joint, genitourinary, miliary, peritoneal, meninges and/or central nervous system (CNS), and all other sites combined. If information from the ICD-9 classification was not available, we used the general information about location of the disease. Case-patients with general location pulmonary disease were allocated to the pulmonary TB group and those with extrapulmonary disease were allocated to the extrapulmonary TB group, site of disease unspecified. Patients for whom no information about the ICD-9 classification was available, and with the general location of combined pulmonary and extrapulmonary disease, were excluded from the analysis.

Each patient was included once in our dataset. If a patient was reported to the register with TB more than once from January 1, 1993, through December 31, 2001, only the information from the first registration was included in the dataset. Patients with a culture diagnosis of *Mycobacterium bovis* and *M. bovis* BCG were excluded as were patients with missing data for age, sex, or nationality

We obtained the total number of inhabitants with Dutch and non-Dutch nationality in the Netherlands from January 1, 1993, through January 1, 2002, from the Central Bureau of Statistics of the Netherlands (*29*). We used these figures as denominators to assess trends in time of the incidence of extrapulmonary and pulmonary TB by Dutch or non-Dutch nationality with Poisson regression. One-way analysis of variance (ANOVA) was used to analyze differences in means of age. We used the χ^2 test to compare the proportions of pulmonary and extrapulmonary TB cases and non parametric tests to compare time to TB diagnosis from immigration for foreign born persons. Differences between pulmonary TB and the 8 separate types of extrapulmonary TB, were tested by using logistic regression, with pulmonary disease as the reference category. The variables age, sex, nationality and HIV status were included in a multivariate logistic regression model to adjust for confounding. Statistical analysis was performed by using SPSS version 11.5 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Between January 1993 and December 2001, 13,943 cases of TB were reported to the Netherlands Tuberculosis Register, 10,688 (76.7%) were culture positive. The 270 case-patients that were reregistrations were excluded. Also excluded were 135 patients who had a diagnosis of *M. bovis* or *M. bovis* BCG infection; 190 patients with missing information about age, sex, or nationality; and 90 patients with a missing ICD-9 classification and combined pulmonary and extrapulmonary disease.

Of the total of 13,258 (95.1%) TB patients available for analysis, 8,216 (62.0%) had pulmonary TB, and 5,042 (38.0%) had an extrapulmonary major site of disease

(Table 1). The proportion with culture confirmation was slightly larger among the pulmonary (71.5%) than among the extrapulmonary TB patients (67.4%) (χ^2 test, p<0.01). The male-to-female ratio was 1.5:1. Male TB patients were slightly younger than female TB patients (ANOVA, p<0.01), the mean age for men was 39.0 years and for women, 40.2 years. Most TB patients (57.1%) were non-Dutch. Of the 7,576 non-Dutch patients, 1,692 (22.3%) were Somali, 1,600 (21.1%) were Asian, 1,267 (16.7%) were Moroccan, and 1,245 (16.4%) were African (other). Asians included 32 different nationalities; the largest number of patients was from Pakistan (243 cases), followed by Indonesia (239 patients) and the People's Republic of China (179 patients). The African (other) group consisted of 48 nationalities with the largest numbers of patients being from Ethiopia (163 patients) and Angola (146 patients). Patients from Somalia, Asia, and Morocco more frequently had a diagnosis of extrapulmonary TB then did Dutch patients, whereas patients from central and eastern Europe had extrapulmonary TB less often (χ^2 test, p<0.01). Non-Dutch patients were significantly younger than Dutch patients (ANOVA p<0.01), the mean ages were 31.7 and 49.8 years, respectively. The male-to-female ratio was 1.4:1 among Dutch patients and 1.5:1 among non-Dutch patients (χ^2 test, p<0.01). Among the non-Dutch, 41.7% of the TB patients had extrapulmonary TB, compared with 33.1% of the Dutch TB patients (p<0.01). No interaction was found between the variables age, nationality, sex, and HIV status.

Between 1993 and 2001 the total number of pulmonary TB cases decreased (rate ratio per year 0.96, 95% confidence interval [CI] 0.95–0.96; p<0.01), especially among the Dutch population (Figure). The total number of extrapulmonary patients showed no significant change in time (rate ratio per year 1.00, 95% CI 0.99–1.02; p = 0.40), but the number of extrapulmonary TB patients with a Dutch nationality decreased (rate ratio per year 0.96, 95% CI 0.94–0.98; p<0.01), while the number of extrapulmonary TB patients with a non-Dutch nationality increased (rate ratio per year 1.06, 95% CI 1.04–1.07; p<0.01).

Extrapulmonary TB was relatively more prevalent among female (45.3%) than among male TB patients (33.1%) (p<0.01). The mean age of extrapulmonary TB patients was slightly higher (40.3 years) than that of pulmonary cases (39.0 years; ANOVA p<0.01). The most frequent types of extrapulmonary TB were lymphatic TB (1,963 cases), pleural TB (1,036 cases), and TB of the bones and/or joints (465 cases) (Table 2). Other sites

Table 1. General characteristics of pulmonary and extrapulmonary tuberculosis (TB) patients in the Netherlands, 1993–2001

	Extrapulmonary TB (%)	Pulmonary TB (%)	Total (%)	p value*
Total	5,042 (38.0)	8,216 (62.0)	13,258 (100)	
Sex				<0.01
Male	2,610 (33.1)	5,285 (66.9)	7,895 (100)	
Female	2,432 (45.3)	2,931 (54.7)	5,363 (100)	
Age groups, y				<0.01
≤14	255 (31.4)	556 (68.6)	811 (100)	
15–24	877 (36.1)	1,553 (63.9)	2,430 (100)	
25–34	1,389 (39.3)	2,149 (60.7)	3,538 (100)	
35–44	810 (38.0)	1,321 (62.0)	2,131 (100)	
45–64	893 (40.9)	1,293 (59.1)	2,186 (100)	
≥65	818 (37.8)	1,344 (62.2)	2,162 (100)	
HIV infection†				<0.01
Yes	173 (34.7)	325 (65.3)	498 (100)	
Unknown	4,869 (38.2)	7,891 (61.8)	12,760 (100)	
Nationality				<0.01
Dutch	1,882 (33.1)	3,800 (66.9)	5,682 (100)	
European, central and eastern	70 (15.1)	395 (84.9)	465 (100)	
Turkish	212 (32.1)	449 (67.9)	661 (100)	
Moroccan	509 (40.2)	758 (59.8)	1,267 (100)	
Somali	997 (58.9)	695 (41.1)	1,692 (100)	
African (other)	456 (36.6)	789 (63.4)	1,245 (100)	
Asian	705 (44.1)	895 (55.9)	1,600 (100)	
Other	211 (32.7)	435 (67.3)	646 (100)	
Culture determination				<0.01
Positive	3,400 (36.7)	5,872 (63.3)	9,275 (100)	
Negative	188 (51.8)	175 (48.2)	363 (100)	
Unknown or not performed	1,454 (40.2)	2,166 (59.8)	3,620 (100)	

*p value (2-sided) for proportions by Pearson χ^2

†HIV testing is not a standard procedure for TB patients in the Netherlands. HIV infection is recorded in the National Tuberculosis Register as one of the response options of the item "impaired immunity." Considered as HIV infected were those cases with a record of impaired immunity due to HIV infection; all others were classified as HIV status unknown.

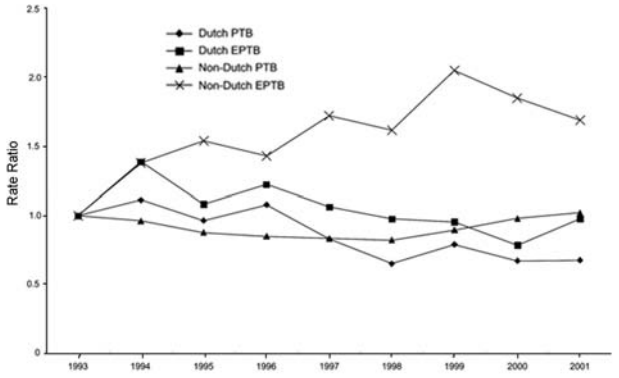

Figure. Relative incidence of pulmonary tuberculosis (PTB) and extrapulmonary tuberculosis (EPTB) among Dutch and non-Dutch residents, the Netherlands, 1993–2001.

accounted for 111 (TB of meninges and/or CNS) to 246 (miliary TB) cases, while for 379 of the extrapulmonary cases, site of disease was not recorded. The proportion of HIV-infected patients ranged from 0.9% (genitourinary TB) to 16.7% (miliary TB), depending on the site of disease (Table 3). The median time since immigration into the Netherlands to diagnosis of TB among non-Dutch was 36.0 months (mean 73.0 months) and varied from 7.9 months in central and eastern Europeans to 9.0 years in Moroccans. Among all non-Dutch pulmonary TB had a shorter time from immigration to diagnosis (median 24.0 months) than extrapulmonary TB (median 44.6 months) (Mann-Whitney U test $p<0.01$).

The genitourinary tract was the site of TB that had the smallest proportion of non-Dutch patients (34.2%) while 80.9% of the patients with peritoneal TB were non-Dutch. Somali TB patients had a strongly increased frequency of peritoneal TB (crude odds ratio 9.4, adjusted odds ratio [AOR] 12, 95% CI 7.6–20), lymphatic TB (crude odds ratio 7.2, AOR 7.8, 95% CI 6.6–9.3) and TB of bones and/or joints (crude odds ratio 3.5, AOR 6.1, 95% CI 4.5–8.3) than the Dutch (Table 4). Asian TB patients had a higher frequency of lymphatic TB (crude odds ratio 4.1, AOR 4.2, 95% CI 3.6–5.0) and peritoneal TB (crude odds ratio 3.2, AOR 3.4, 95% CI 2.0–5.9) than the Dutch. The proportion of TB of the bones and or joints, genitourinary TB, and miliary TB compared to pulmonary TB increased with the age of the patients. Both the age groups ≤14 years

Table 2. Distribution of total tuberculosis (TB) and EPTB by nationality and culture determination in the Netherlands, 1993–2001*†

	Total TB‡ (%)	Total EPTB (%)	Lymphatic (%)	Pleural (%)	Bones and joints (%)	Miliary (%)	Genitourinary (%)	Peritoneal (%)	Meninges and CNS (%)	Other EPTB (%)
Total	13,258 (100)	5,042 (38.0)	1,963 (14.8)	1,036 (7.8)	465 (3.5)	246 (1.9)	226 (1.7)	178 (1.3)	111 (0.8)	438 (3.3)
Nationality										
Dutch	5,682 (100)	1,882 (33.1)	441 (3.3)	592 (4.5)	188 (1.4)	136 (1.0)	147 (1.1)	34 (0.3)	52 (0.4)	133 (1.0)
European (central and eastern)	465 (100)	70 (15.1)	23 (4.9)	26 (5.6)	1 (0.2)	7 (1.5)	3 (0.6)	1 (0.2)	0	4 (0.9)
Turkish	661 (100)	212 (32.1)	88 (13.3)	45 (6.8)	7 (1.1)	4 (0.6)	12 (1.8)	7 (1.1)	3 (0.5)	24 (3.6)
Moroccan	1,267 (100)	509 (40.2)	233 (18.4)	87 (6.9)	29 (2.3)	14 (1.1)	24 (1.9)	29 (2.3)	12 (0.9)	46 (3.6)
Somali	1,692 (100)	997 (58.9)	497 (29.4)	84 (5.0)	107 (6.3)	22 (1.3)	11 (0.7)	58 (3.4)	20 (1.2)	110 (6.5)
African (other)	1,245 (100)	456 (36.6)	189 (15.2)	83 (6.7)	43 (3.5)	32 (2.6)	12 (1.0)	12 (1.0)	5 (0.4)	49 (3.9)
Asian	1,600 (100)	705 (44.1)	407 (25.4)	75 (4.7)	64 (4.0)	19 (1.2)	9 (0.6)	27 (1.7)	16 (1.0)	57 (3.6)
Other	646 (100)	211 (32.7)	85 (13.2)	44 (6.8)	26 (4.0)	12 (1.9)	8 (1.2)	10 (1.5)	3 (0.5)	15 (2.3)
Culture determination										
Positive	9,275 (100)	3,400 (36.7)	1,402 (15.1)	683 (7.4)	362 (3.9)	185 (2.0)	167 (1.8)	141 (1.5)	74 (0.8)	288 (3.1)
Negative	363 (100)	188 (51.8)	80 (22.0)	59 (16.3)	13 (3.6)	2 (0.6)	5 (1.4)	3 (0.8)	6 (1.7)	16 (4.4)
Unknown or not performed	3,620 (100)	1,454 (40.2)	481 (13.3)	294 (8.1)	90 (2.5)	59 (1.6)	54 (1.5)	34 (0.9)	31 (0.9)	134 (3.7)

*EPTB, extrapulmonary TB; CNS, central nervous system.
†The denominator for percentages is the total TB cases in the row.
‡Total TB includes both PTB and EPTB.

Table 3. Distribution of total tuberculosis (TB) and EPTB by sex, age group, and HIV diagnosis category in the Netherlands, 1993–2001*†

	Total TB‡ (%)	Total EPTB (%)	Lymphatic (%)	Pleural (%)	Bones and joints (%)	Miliary (%)	Genitourinary (%)	Peritoneal (%)	Meninges and CNS (%)	Other EPTB (%)
Total	13,258 (100)	5,042 (38.0)	1,963 (14.8)	1,036 (7.8)	465 (3.5)	246 (1.9)	226 (1.7)	178 (1.3)	111 (0.8)	438 (3.3)
Sex										
Male	7,895 (100)	2,610 (33.1)	904 (11.5)	676 (8.6)	224 (2.8)	136 (1.7)	121 (1.5)	78 (1.0)	69 (0.9)	219 (2.8)
Female	5,363 (100)	2,432 (45.3)	1,059 (19.7)	360 (6.7)	241 (4.5)	110 (2.1)	105 (2.0)	100 (1.9)	42 (0.8)	219 (4.1)
Age group, y										
≤14	811 (100)	255 (31.4)	133 (16.4)	32 (3.9)	14 (1.7)	6 (0.7)	0	3 (0.4)	13 (1.6)	23 (2.8)
15–24	2,430 (100)	877 (36.1)	373 (15.3)	204 (8.4)	66 (2.7)	26 (1.1)	10 (0.4)	41 (1.7)	10 (0.4)	82 (3.4)
25–34	3,538 (100)	1,389 (39.3)	608 (17.2)	312 (8.8)	105 (3.0)	40 (1.1)	30 (0.8)	50 (1.4)	31 (0.9)	121 (3.4)
35–44	2,131 (100)	810 (38.0)	347 (16.3)	140 (6.6)	75 (3.5)	34 (1.6)	37 (1.7)	37 (1.7)	13 (0.6)	77 (3.6)
45–64	2,186 (100)	893 (40.9)	301 (13.8)	165 (7.5)	97 (4.4)	45 (2.1)	75 (3.4)	34 (1.6)	28 (1.3)	77 (3.5)
≥65	2,162 (100)	818 (37.8)	201 (9.3)	183 (8.5)	108 (5.0)	95 (4.4)	74 (3.4)	13 (0.6)	16 (0.7)	58 (2.7)
HIV infection§										
Yes	498 (100)	173 (34.7)	60 (12.0)	18 (3.6)	9 (1.8)	41 (8.2)	2 (0.4)	8 (1.6)	10 (2.0)	21 (4.2)
Unknown	12,760 (100)	4,869 (100)	1,903 (14.9)	1,018 (8.0)	456 (3.6)	205 (1.6)	224 (1.8)	170 (1.3)	101 (0.8)	417 (3.3)

*EPTB, extrapulmonary TB; CNS, central nervous system.
†The denominator for percentages is the total TB cases in the row.
‡Total TB includes both PTB and EPTB.
§HIV testing is not a standard procedure for TB patients in the Netherlands. HIV infection is recorded in the National Tuberculosis Register as one of the response options of the item "impaired immunity." Considered as HIV infected were those cases with a record of impaired immunity due to HIV infection; all others were classified as HIV status unknown.

and 45–64 years were associated with TB of the meninges and/or CNS, in univariate analysis (crude odds ratio 3.9 and 1.8, respectively). When the study population was stratified in ≤14 years and ≥15 years age groups, all types of extrapulmonary TB were associated with the oldest age group, except for TB of the meninges and/or CNS, which was weakly associated with the youngest age group (AOR 1.7, 95% CI 0.9–3.1). HIV infection was positively associated with miliary TB (AOR 7.4, 95% CI 4.9–11) and TB of the meninges and/or CNS (AOR 3.3, 95% CI 1.6–6.7) and negatively associated with pleural and genitourinary TB.

Discussion

In the Netherlands, the number of inhabitants with a non-Western ethnic background increased over the past 2 decades (*23*). The Netherlands is likely to remain an immigration destination for persons from non-Western countries, although changes in immigration laws can change this situation (*23*,*24*). We assessed the effect of these immigration patterns on the incidence of pulmonary and extrapulmonary TB.

Between 1993 and 2001, the number of pulmonary TB cases per year declined, whereas the number of extrapulmonary TB cases remained stable, thus showing a proportional increase. This trend is similar to the trend observed in studies in the United States (*6*,*7*). Our study shows that a non-Dutch nationality, especially Somali and Asian, was positively associated with extrapulmonary TB when compared with the results for pulmonary TB. This finding suggests that the most likely explanation for the proportional increase of extrapulmonary TB is the growth of the number of inhabitants with a non-Western ethnic background.

Our analysis showed that persons from non-Western national groups, especially Somalis, Asians, and Moroccans, were more likely to receive a diagnosis of most types of extrapulmonary TB than Dutch nationals. When looking at the absolute number of patients with lymphatic and peritoneal TB exclusively, patients with Somali nationality even outnumbered Dutch patients (497 and 58 vs 441 and 34, respectively). In agreement with the literature, we found a strongly positive association between Somali nationality and lymphatic and bone and/or joint TB (*18*,*27*) and between Asian nationalities and lymphatic TB (*6*,*10*,*11*,*16*). Our study demonstrated a statistically significant, strong, positive association between peritoneal TB and Somali, Moroccan, or Asian nationality. In contrast,

Table 4. Risk factors for 8 types of extrapulmonary tuberculosis (TB) versus pulmonary TB*†

	Lymphatic (n = 1,963)	Pleural (n = 1,036)	Bones and joints (n = 465)	Miliary (n = 246)	Genitourinary (n = 226)	Peritoneal (n =178)	Meninges and CNS (n = 111)	Other EPTB sites (n = 438)
Sex								
Male	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Female	**2.22 (2.00–2.46)**	0.94 (0.82–1.08)	**1.95 (1.61–2.37)**	**1.56 (1.20–2.03)**	**1.70 (1.29–2.22)**	**2.55 (1.87–3.46)**	1.08 (0.73–1.60)	**1.93 (1.59–2.36)**
Age group, y								
≤14	**0.68 (0.52–0.89)**	**0.52 (0.35–0.77)**	**0.16 (0.09–0.29)**	**0.10 (0.04–0.23)**	–	**0.18 (0.05–0.66)**	1.54 (0.70–3.36)	**0.44 (0.26–0.75)**
15–24	**0.63 (0.51–0.78)**	**1.34 (1.06–1.68)**	**0.27 (0.18–0.38)**	**0.14 (0.08–0.23)**	**0.12 (0.06–0.25)**	0.90 (0.45–1.81)	0.43 (0.18–1.01)	**0.54 (0.37–0.81)**
25–34	0.86 (0.70–1.05)	**1.51 (1.22–1.86)**	**0.36 (0.26–0.50)**	**0.14 (0.09–0.22)**	**0.29 (0.18–0.47)**	0.94 (0.48–1.85)	0.97 (0.49–1.90)	**0.68 (0.47–0.98)**
35– 44	1.03 (0.83–1.27)	1.05 (0.82–1.34)	**0.56 (0.40–0.78)**	**0.20 (0.13–0.33)**	**0.62 (0.40–0.95)**	1.58 (0.80–3.13)	0.70 (0.32–1.53)	0.92 (0.63–1.35)
45–64	1.11 (0.90–1.37)	1.08 (0.86–1.35)	0.85 (0.63–1.15)	**0.41 (0.28–0.60)**	1.16 (0.83–1.64)	1.85 (0.95–3.62)	1.71 (0.91–3.23)	1.13 (0.78–1.63)
≥65	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
HIV infection‡								
Yes	0.92 (0.68–1.24)	**0.40 (0.25–0.65)**	0.52 (0.26–1.03)	**7.36 (4.88–11.12)**	**0.25 (0.06–1.04)**	1.41 (0.66–3.01)	**3.29 (1.63–6.65)**	1.36 (0.85–2.19)
Unknown	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Nationality								
Dutch	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
European (central and eastern)	**0.57 (0.37–0.88)**	**0.37 (0.25–0.56)**	**0.08 (0.01–0.54)**	1.06 (0.48–2.33)	0.31 (0.10–0.98)	0.31 (0.04–2.32)	–	**0.35 (0.13–0.96)**
Turkish	**2.01 (1.55–2.61)**	**0.55 (0.40–0.76)**	0.53 (0.25–1.15)	0.64 (0.23–1.77)	1.27 (0.68–2.38)	2.19 (0.95–5.09)	0.63 (0.19–2.07)	**2.01 (1.26–3.19)**
Moroccan	**2.98 (2.47–3.60)**	**0.67 (0.53–0.86)**	1.11 (0.73–1.68)	1.12 (0.63–2.00)	1.23 (0.78–1.96)	**4.71 (2.78–7.97)**	1.31 (0.68–2.52)	**2.08 (1.45–2.99)**
Somali	**7.82 (6.56–9.31)**	**0.68 (0.53–0.88)**	**6.10 (4.51–8.26)**	**2.75 (1.61–4.67)**	1.14 (0.59–2.22)	**12.47 (7.64–20.36)**	**2.82 (1.57–5.07)**	**6.39 (4.69–8.70)**
African (other)	**2.79 (2.27–3.42)**	**0.61 (0.47–0.79)**	**2.32 (1.59–3.39)**	**2.28 (1.41–3.66)**	1.09 (0.58–2.07)	**2.21 (1.09–4.46)**	0.53 (0.20–1.39)	**2.48 (1.71–3.60)**
Asian	**4.19 (3.55–4.96)**	**0.47 (0.36–0.61)**	**2.06 (1.50–2.83)**	1.28 (0.77–2.15)	**0.42 (0.21–0.84)**	**3.44 (2.01–5.91)**	1.63 (0.89–2.95)	**2.09 (1.49–2.94)**
Other	**1.78 (1.37–2.31)**	**0.63 (0.46–0.88)**	**1.70 (1.08–2.59)**	1.03 (0.55–1.94)	0.67 (0.32–1.39)	**2.44 (1.17–5.07)**	0.47 (0.14–1.54)	1.05 (0.60–1.83)

*EPTB, extrapulmonary tuberculosis; PTB, pulmonary TB.
†In the calculation of odds ratios for patients, patients with PTB served as a reference group. The odds ratios were adjusted for all variables used in the model. **Boldface** figures denote significant odds ratios.
‡HIV testing is not a standard procedure for TB patients in the Netherlands. HIV infection is recorded in the National Tuberculosis Register as one of the response options of the item "impaired immunity." Considered as HIV infected were those cases with a record of impaired immunity due to HIV infection; all others were classified as HIV status unknown..

pleural TB was significant negatively associated with the non-Dutch patients when results were compared with those of the Dutch patients, a finding that we cannot explain.

Several explanations are possible for the association of extrapulmonary TB and a non-Dutch nationality. Non-Dutch persons may have a higher frequency of extrapulmonary TB due to an impaired immunity caused by factors such as vitamin D deficiency (*11*,*16*,*30*,*31*), dietary changes (*16*), and restricted social conditions (*16*,*18*), which cause an endogenous TB infection to reactivate from extrapulmonary or pulmonary sites. Also genetic factors (*32*), for example the presence of polymorphism of the *NRAMP1* gene (*33*,*34*) may contribute to differences in the susceptibility to acquire extrapulmonary TB. Furthermore, *M. tuberculosis* strains circulating outside the Netherlands may be genetically different from those circulating in the Netherlands and cause more extrapulmonary TB.

A limitation in our study is the use of different methods of case finding, since immigrants from countries with a high prevalence are screened for pulmonary TB, but not for extrapulmonary TB (*26*). This circumstance could lead to a possible underestimation of extrapulmonary TB among immigrants, which would make the relationships found in this study even stronger. On the other hand, selection bias is possible when physicians disproportionately suspect and diagnose extrapulmonary TB among certain groups of patients such as non-Western patients. By including 97.0% of all reported new cases of TB in the analysis (13,258 of 13,673 new cases), we tried to minimize bias on inclusion in the study. Fewer patients with extrapulmonary TB had a positive culture than did patients with pulmonary TB (67.4% vs 71.5%). Thus patients with extrapulmonary TB may be misdiagnosed. However, a

sensitivity analysis, which included only the culture-positive cases, did not change the conclusions of the logistic regression analysis. Also misdiagnosis of *M. bovis* as *M. tuberculosis* may have occurred in cases in which no culture result was available. Of the 114 *M. bovis* patients excluded from the analysis, all locations of TB were identified: 7 patients had pleural TB, 30 had lymphatic TB, 2 had meningeal TB, 3 had peritoneal TB, 8 had TB of bones and/or joints, 8 had genitourinary TB, 5 had miliary TB, 5 had other extrapulmonary TB, 42 had pulmonary TB, and 4 had extrapulmonary TB not specified.

It should be noted that nationality in the NTR is not always recorded based on passport; sometimes ethnic background is used. Furthermore, a difference in the definition of extrapulmonary TB used in different studies complicates mutual comparison.

Many studies suggest that HIV-induced immunosuppression is associated with extrapulmonary disease (*7*,*35*–*37*), especially with lymphatic and miliary TB (*38*,*39*). In the Netherlands, HIV testing is not a standard procedure for TB patients, which may have led to a possible selection bias in our study. A possible explanation for the absence of associations between HIV infection and most types of extrapulmonary TB in our study can be found in the introduction of the highly active antiretroviral therapy (HAART) in 1996 (*40*). HIV-infected patients treated with HAART will have less impaired immunity (*39*).

Diagnosing extrapulmonary TB can be difficult, so a high index of suspicion remains important. In immigrants from countries with highly endemic TB, a medical history, physical examination, basic laboratory tests, and chest radiograph can lead to a diagnosis. Since TB can occur in all organs, further examination depends on the possible site of infection.

In summary, our analysis showed that there is a proportional increase of extrapulmonary TB in the Netherlands. The growth of the number of inhabitants with a non-Western ethnic background in the Netherlands explains the proportional growth of extrapulmonary TB. Increased awareness among physicians about the changing clinical picture and up-to date knowledge about diagnosis of TB is warranted.

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Risk for Tuberculosis among Children

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Contacts of adults with tuberculosis (TB) are at risk for infection. Tests based on interferon-γ (IFN-γ) expression in response to *Mycobacterium tuberculosis* antigens may be more sensitive than the tuberculin skin test (TST). Risk for infection was assessed by using TST and an IFN-γ-based assay (QuantiFERON Gold in Tube [QFT-IT] test) for 207 children in Nigeria in 1 of 3 groups: contact with adults with smear-positive TB, contact with adults with smear-negative TB, and controls. For these 3 groups, respectively, TST results were >10 mm for 38 (49%) of 78, 13 (16%) of 83, and 6 (13%) of 46 and QFT-IT positive for 53 (74%) of 72, 8 (10%) of 81, and 4 (10.3%) of 39 ($p<0.01$). Most test discrepancies were TST negative; QFT-IT positive if in contact with TB-positive persons; and TST positive, QFT-IT negative if in contact with TB-negative persons or controls. TST may underestimate risk for infection with TB in children.

Tuberculosis (TB) is the most important infectious cause of adult deaths, and persons with acid-fast bacilli (AFB) in their sputum are the most infectious group in the community (*1*,*2*). Children exposed to adults with smear-positive pulmonary TB have a high risk for infection, and this risk increases with the degree of contact (*3*,*4*). In countries with a high incidence of TB, risk for infection among children in contact with adults with TB is 30%–50%, which is much higher than that reported by industrialized countries (*5*,*6*). However, these risk estimates were established with the tuberculin skin test (TST), which has several limitations. Children vaccinated with *Mycobacterium bovis* BCG or infected with mycobacteria other than *M. tuberculosis* can have false-positive TST reactions, and those with malnutrition, measles, and HIV or other infections often have false-negative reactions (*7*). In areas with a high incidence of TB, low sensitivity and specificity of the TST may result in either overestimation or underestimation of the risk for transmission.

New tests based on the expression of interferon-gamma (IFN-γ) by sensitized lymphocytes in response to specific *M. tuberculosis* antigens (e.g., early secretory protein 6 [ESAT-6] and culture filtrate protein 10 [CFP-10]) appear to be more specific (*8*) and sensitive (*9*) than the TST in identifying latent and active TB. Although a test with these characteristics could have enormous practical implications for improving management of children at high risk, most studies have focused on adults in countries with low incidence of TB.

This study assesses the risk for latent TB infection among young household contacts of adults with pulmonary TB in Nigeria, a country with a high incidence of TB. We compared the TST and the QuantiFERON TB Gold in Tube (QFT-IT) (Cellestis International, Carnegie, Victoria, Australia) test.

Materials and Methods

We conducted a cross-sectional study of children in contact with adults who had pulmonary TB in Abuja, Nigeria. Incidence of TB in Nigeria is among the highest in Africa, with and estimated 293 cases/100,000 persons per year, of whom 126/100,000 are smear positive. Study children were identified by visiting the households of adults whose TB had been diagnosed at enrollment in a separate study of TB diagnosis from September 2003 to November 2004 (*10*). These index adults had undergone HIV counseling and testing and had a diagnosis of TB. Briefly, screening for HIV was done with ImmunoComb HIV1 & 2 BiSpot kits (Orgenics, Yavne, Israel) for all

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patients enrolled in this study. Sputum samples were collected for 2 days, and smear microscopy and BACTEC culture (Becton Dickinson, Sparks, MD, USA) were conducted by trained staff at Zankli TB Research Laboratory in Abuja. Results of smear microscopy were recorded according to the grading system of the International Union Against Tuberculosis and Lung Diseases (*11*) as –, scanty, +, ++, or +++ AFB. Since all adults enrolled in the study had positive cultures, they were classified as having smear-positive or smear-negative TB.

Home visits were conducted between March and May 2005, which was ≥12 weeks after diagnosis of the index case of TB. Eligible children were defined as any relative in the household <15 years of age who ate food prepared in the same cooking facilities as the index patient. During a home visit, a list of the children in the family was obtained, and ≤5 of these children were selected randomly to participate in the study. The parents were interviewed by using a standardized questionnaire concerning medical history, degree of contact, and characteristics of the household. Information was also obtained regarding BCG vaccination, weight, height, and clinical signs of TB. The HIV status of the children was not known because there was no medical reason for obtaining it. HIV status of the parents was assessed as part of their routine investigation for diagnosis of TB.

A separate group of children <15 years of age who were not exposed to adults with TB was selected to assess the prevalence of asymptomatic infections in the community. These control children were selected by visiting households situated at least 100 m from an index patient's household to avoid the possibility of cross-infection, maintain anonymity, and use the procedures steps as in the group not exposed to TB. Children were selected after ascertaining that adults did not have symptoms of TB.

After examination, all children were tested with the TST and QFT-IT test. The QFT-IT test uses overlapping synthetic peptides (ESAT-6 and CFT-10) that are specific for *M. tuberculosis*. For the TST, 10 U of purified protein derivative (Chiron Vaccines Evans, Liverpool, UK), equivalent to 5 IU tuberculin were injected by using the Mantoux method (available from http://www.immunisation.nhs.uk/files/PPD_difference.pdf) on the day of the initial visit. TST readings were obtained by using the palpation method 48–72 hours later (*12*) and were classified as negative if induration was <5 mm, intermediate if 5–9 mm, and positive if ≥10 mm. Children with symptoms compatible with TB were referred to the hospitals for further assessment and treatment. Parents of children with a positive TST result but normal examination results were given advice and registered for follow-up to allow monitoring of symptoms. Chemoprophylaxis is not routinely given in Nigeria.

The QFT-IT test was performed according to the manufacturer's instructions. Briefly, 1 mL of blood was drawn into vacutainer (Becton Dickinson) tubes coated with either saline (negative control tube) or ESAT-6, CFP-10, and TB 7.7 peptides (*M. tuberculosis*–specific antigen tubes), transported to the laboratory in Abuja 2–6 hours after collection, and incubated overnight at 37°C. The tubes were then centrifuged, and the supernatant plasma was harvested and stored at −80°C until transported to Hvidovre Hospital in Copenhagen, Denmark for IFN-γ analysis with an ELISA. Technicians performing ELISAs were unaware of clinical data, including TST status. IFN-γ detected in the saline control tube was subtracted from that in the TB antigen tube. Samples with a difference ≥0.35 IU/mL IFN-γ after stimulation with *M. tuberculosis*–specific antigens were considered positive; samples with differences <0.35 IU/mL were considered negative. Calculations were performed with software provided by the manufacturer. Samples from 33 children who had high background IFN-γ levels (control value >0.7 IU/mL) were retested. These duplicate tests provided identical results and were interpreted according to the manufacturer's guidelines.

Data were analyzed by using EpiInfo version 3.2.2 (Centers for Disease Control and Prevention, Atlanta, GA, USA). The proportion (95% confidence interval) of children with positive test results was calculated according to the smear status of the index patients. Student *t* tests were used for comparing means of continuous variables, χ^2 tests for categorical variables, and nonparametric tests for continuous variables with skewed distributions. We analyzed our results by using a generalized estimation equation because of the likelihood of clustering of cases in families. However, the precision of the estimates did not change and they were not used.

Agreement between TST and QFT-IT test results was analyzed by using kappa statistics. Disagreement between test results was also tested according to risk for infection from index patients. Children in contact with adults with smear-negative TB and community controls were classified as being at low risk for infection. Children in contact with smear-positive adults (scanty or more AFB) were classified as high risk.

Written informed consent was obtained from parents or guardians of children. Illiterate parents were asked to provide oral consent in the presence of a witness. The study protocol was approved by the Research Ethics Committee of the Liverpool School of Tropical Medicine and the Zankli Ethical and Research Review Board in Abuja, Nigeria.

Results

Sixty index adult case-patients living in 56 households were visited. Of these, 27 (45%) were male, 27 (45%)

were smear-positive, and 33 (55%) were smear-negative. The mean duration between their initial TB diagnosis and follow-up household visits was 54 weeks (range 27–88 weeks). All smear-positive index adults had completed or were receiving treatment, 15 (56%) were co-infected with HIV, and none had died. Eighteen (55%) of the 33 adults with smear-negative TB had not started treatment, 5 (15%) had completed treatment, 4 (12%) had defaulted, and 6 (18%) had died. Thirteen (39%) were co-infected with HIV, and 10 (33%) has an unknown HIV status. According to national policy, the index case-patients who had not started treatment were registered for treatment during the course of this study.

Figure 1 shows a flow chart of the study participants. Of 207 children enrolled in the study, 83 were in contact with adults with smear-negative TB, 78 were in contact with adults with smear-positive TB, and 46 were community controls. A summary of their characteristics is shown in Table 1. Their mean (standard deviation) age was 7.4 (3.8) years (range 1–14 years) and 95 (46%) were male. Previous BCG vaccination was reported for 187 (90%) children, but BCG scars were present in a lower proportion of children in contact with smear-positive adults. This latter group of children also reported slightly more contact time and shared bedrooms more frequently with index-case adults than children in contact with patients with smear-negative TB ($p<0.01$ for both). Two (1%) children in contact with adults with smear-positive TB had cough for >3 weeks and were given therapy for TB.

TST readings were available for 193 (93%) children, and positivity varied according to the number of AFB in sputum of the adults (Figure 2). Similar proportions of control children (6/48 [15%]) and children in contact with adults with smear-negative TB (13/80 [16%]) were TST positive. A larger proportion (38/78 [53%]) of children in contact with adults with smear-positive TB were TST positive ($p<0.001$).

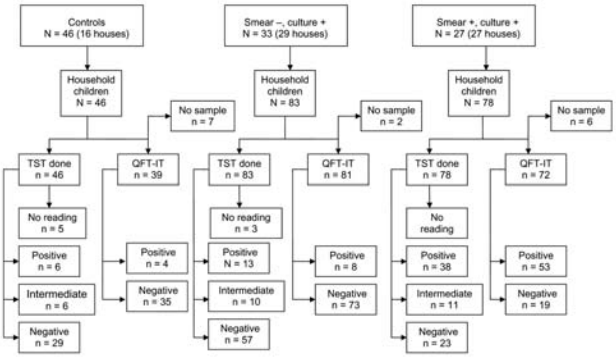

Figure 1. Flow chart of the study participants. TST, tuberculin skin test; QFT-IT, QuantiFERON Gold in Tube test.

The relationship of the TST result with age is shown in Figure 3A. The proportion of children with positive TST results increased with age in both control children and in children in contact with adults with smear-negative TB. However, age was not a risk factor for children of adults with smear-positive TB because all children in this group had a high frequency of positive results independent of age.

QFT-IT test responses also varied according to the characteristics of the adults. Positive results were obtained for 10% (4/39) of community controls, 10% (98/81) of children in contact with adults with smear-negative TB, and 74% (53/72) of children in contact with adults with smear-positive TB (Figure 2). As was found with TST, the proportion of children with positive QFT-IT test results increased with age in community controls and in children of adults with smear-negative TB, but was high across all ages in children in contact with adults with smear-positive TB (Figure 3B).

Table 1. Characteristics of children by study group*

Characteristic	Controls (N = 46), no. (%)	Smear status of index case-patients: Smear-negative tuberculosis (n = 83), no. (%)	Smear status of index case-patients: Smear-positive tuberculosis (n = 78), no. (%)	p value†
Mean (SD) age, y	6.2 (3.5)	7.5 (3.8)	7.9 (3.8)	0.04
Male/female (% male)	22/24 (48)	39/44 (47)	34/44 (44)	NS
History of *Mycobacterium bovis* BCG	42 (91)	77 (93)	68 (87)	NS
BCG scar present	18 (39)	39 (47)	19 (24)	0.01
Relationship to index case-patient				
Son or daughter	NA	47 (57)	59 (76)	<0.01
Grandchild	NA	7 (8)	0	
Sibling	NA	9 (11)	15 (19)	
Niece or nephew	NA	14 (17)	4 (5)	
Other	NA	6 (7)	0	
Mean (SD) hours in contact per day	NA	9.6 (4.9)	11.2 (5)	0.04
Shares bedroom with index case	NA	28 (34)	50 (64)	<0.01
Mean (SD) number people per bedroom	4.4 (1.8)	3.3 (1.2)	4.3 (2)	<0.01

*SD, standard deviation; NS, not significant; NA, not applicable.
†Comparison of proportions and means between the 3 groups unless data do not apply to the control group.

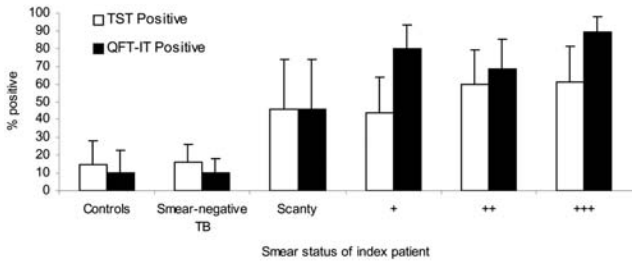

Figure 2. Proportion of children with positive tuberculin skin test (TST) (>10 mm) and QuantiFERON Gold in Tube (QFT-IT) test results, by adult smear positivity. Error bars show 95% confidence intervals.

An association was found between the QFT-IT test results and bacillary load in the sputum of the adults. Forty-five percent (4/11), 80% (16/20), 68% (15/22), and 90% (17/19) of children in contact with adults with scanty, +, ++, and +++ AFB, respectively, had positive results from the QFT-IT test ($p = 0.03$), as shown in Figure 2.

Comparison of test results in children with low and high risks for infection is shown in Table 2. A total of 113 children with low risk for infection and 66 with high risk for infection had both TST and QFT-IT results available. For this purpose only, children with intermediate TST responses were grouped with children with negative TST results. There was concurrence in 84 (74%) of the TST and QFT-IT test results in the low-risk group and 49 (74%) of the test results in the high-risk group (κ 0.246 and 0.498, respectively, $p<0.05$). In the low-risk group, most children with discordant results had positive or intermediate TST results but negative QFT-IT test results. However, in the high-risk group, most children with discordant results had negative or intermediate TST and positive QFT-IT test results.

Discussion

TB infection often occurs in childhood, and children are often infected within the home (*5*). Children with latent TB infections have a high risk of developing overt disease and children <5 years of age are the most vulnerable. In industrialized countries, assays that measure IFN-γ expression in response to specific *M. tuberculosis* antigens can identify infections in exposed adults as well as in children (*13*,*14*). However, few studies have evaluated the performance of these assays in children in countries endemic for TB, and these studies have mainly focused on children with active TB (*15*,*16*). The current study assessed the risk for infection as determined by TST and QFT-IT test in children exposed to adults with microbiologically confirmed TB.

In our study, the risk for infection was largely defined by the age of the children and the smear status of the adults. In community controls, the overall rate of infection ranged from 10% (as measured by QFT-IT test) to 15% (as measured by TST) and was consistent with rates previously reported from other parts of Africa (*17*,*18*). Children in contact with adults with smear-negative TB had results similar to those of community controls, a finding consistent with the current perception that adults with smear-negative TB are less infectious (*3*,*19*). The risk for infection after exposure to adults with smear-positive TB, as determined by TST (53%), corresponds to previous values of 30% to 50% in high-incidence countries (*5*,*6*). However, the higher rate of infection determined by QFT-IT test (74%) suggests that the TST might underestimate the risk for infection for contacts of adults with smear-positive TB.

In contrast to the pattern observed for community controls, TST and QFT-IT test responses did not increase with age for contacts of smear-positive cases, suggesting that children of all ages are at high risk for infection in these households Our findings are consistent with those of previous studies of mixed-age populations that used different versions of IFN-γ tests and reported infection rates of 30% to 70% for household contacts (*16*,*20*,*21*) and an association between proximity of an index patient and positive IFN-γ responses (*13*,*14*,*20*). We also observed a trend of increasing TST and QFT-IT responders with increasing

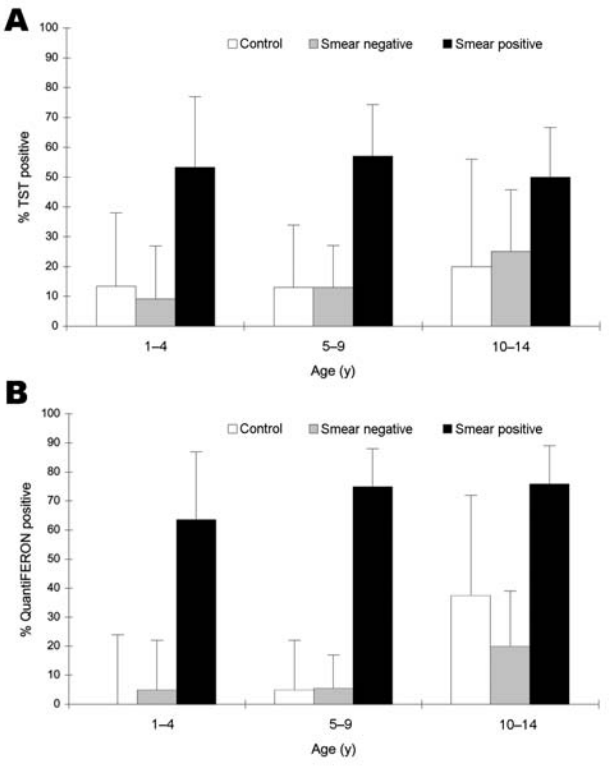

Figure 3. Proportion of children with positive tuberculin skin test results, by age. A) Tuberculin skin test (TST) (>10 mm). B) QuantiFERON Gold in Tube (QFT-IT) test. Error bars show 95% confidence intervals.

Table 2. Agreement between TST and QFT-IT results in children at high risk and low risk for tuberculosis (TB)*

	QFT-IT	
TST	Positive	Negative
Low-risk group		
Positive	6	12
Intermediate	2	13
Negative	2	78
High-risk group		
Positive	34	2
Intermediate	9	0
Negative	6	15

*TST, tuberculin skin test; QFT-IT, QuantiFERON Gold in Tube test; low risk, children in contact with adults with smear-negative TB and community controls; high risk, children in contact with smear-positive adults. TST result was negative if <5 mm, intermediate if 5–9 mm, and positive if ≥10 mm. TST and QFN-IT test results were missing in 13 children in the low-risk group and 12 children in the high-risk group

numbers of bacilli in sputum, which may indicate a dose-response relationship. This relationship has been previously reported for the TST (*5*,*20*) but is a new finding for QFT-IT test. We did not find any association between the HIV status of adults and TST and QFT-IT test results of children, but larger studies should be conducted to establish whether a relationship exists between HIV and transmission of TB within the household.

Only a few studies have assessed antigen-specific IFN-γ responses in African children with TB. Two studies found a high sensitivity (≈83%) for detection of IFN-γ in children with confirmed TB by using the ELISPOT technique (*15*,*16*). Children with active TB were more likely to have positive responses than asymptomatic children, and the assays seemed more sensitive than TST independent of age, HIV status, and malnutrition (*15*). Nicol et al. found that 50% of 26 South African children living in households with sputum-positive TB patients had TB-positive results in the IFN-γ test (*16*).

Previous studies that used with IFN-γ assays have reported a high specificity for ESAT-6 and CFP-10 (*8*,*9*,*22*). However, without a standard to determine latent TB infections and discriminate between true- and false-positive QFT-IT test results, we analyzed the agreement between the TST and QFT-IT test results to determine whether any disagreement had a different pattern in children with high and low risks for infection. In children at low risk (controls and contacts of adults with smear-negative TB), most disagreement was because TST results were positive and QFT-IT test results were negative. In contrast, in children at high risk (in contact with adults with smear-positive TB), most disagreement was because TST results were negative and QFT-IT test results were positive. This finding suggests that the pattern of disagreement was not random and may have reflected a higher sensitivity of the QFT-IT test. The correlation between TST and IFN-γ test results has been inconsistent; studies have shown good agreement in low-risk BCG unvaccinated groups (*13*) and poor agreement in BCG-vaccinated groups (*9*). These differences may reflect differences in the incidence of TB (and thus the risk for infection), laboratory methods, and cut-off values.

The advantage of new antigen-specific IFN-γ tests is their supposed high specificity. In adults, IFN-γ responses are repressed in patients with active TB (*23*) and increase after initiation of therapy for TB (*24*). In cattle, an ESAT-6-specific IFN-γ assay is reported to be a good predictor of disease severity (*25*). In humans (adults), this assay may indicate recovery from disease (*26*). It is thus plausible that the pattern of IFN-γ expression after infection is different from the pattern of TST responses over time. Thus, the former pattern might be associated with disease activity and the latter with identification of history of infection. If true, children with recent, active infections would have different IFN-γ responses than children with latent infections.

Despite increased risk of developing active TB for recent TST converters (*7*,*27*), a similar association has not yet been demonstrated for the *M. tuberculosis*–specific tests. Preliminary reports suggest that quantitative measurements of a patient's response may correlate with disease progression, and results from a small study in Ethiopia suggest that ESAT-6 responses might be predictive of the risk for developing active TB in subsequent months (*28*). Studies formally demonstrating such association are necessary.

A proportion of our children (≈15%, data not shown) had high IFN-γ background levels that could not be explained by technical errors. The reason for this high background may be in vivo activation of T lymphocytes specific for other infections such as malaria and other parasitic or viral diseases, which are highly prevalent in the study area. Additional studies are needed to assess the reliability of IFN-γ assays in areas with high incidence of infectious diseases. Current tests also require techniques and skills that are rarely available in the areas where most patients with TB contact the health services (*29*). Thus, more portable and simpler tests are needed to improve their applicability. Similarly, the current cost of the tests is too expensive for most developing countries (≈US $20/test). If these tests prove to be useful, marketing strategies would be needed to substantially reduce their costs.

In conclusion, exposure to adults with smear-positive TB was the most important risk factor for transmission within households. Infection rates for children exposed to adults with smear-negative TB were similar to that for community controls. The QFT-IT test detects latent TB infection more often than the TST in children of sputum-positive parents in Nigeria.

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Multidrug-resistant Tuberculosis Management in Resource-limited Settings

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Evidence of successful management of multidrug-resistant tuberculosis (MDRTB) is mainly generated from referral hospitals in high-income countries. We evaluate the management of MDRTB in 5 resource-limited countries: Estonia, Latvia, Peru, the Philippines, and the Russian Federation. All projects were approved by the Green Light Committee for access to quality-assured second-line drugs provided at reduced price for MDRTB management. Of 1,047 MDRTB patients evaluated, 119 (11%) were new, and 928 (89%) had received treatment previously. More than 50% of previously treated patients had received both first- and second-line drugs, and 65% of all patients had infections that were resistant to both first- and second-line drugs. Treatment was successful in 70% of all patients, but success rate was higher among new (77%) than among previously treated patients (69%). In resource-limited settings, treatment of MDRTB provided through, or in collaboration with, national TB programs can yield results similar to those from wealthier settings.

Multidrug-resistant tuberculosis (MDRTB), defined as TB resistant to at least isoniazid and rifampin, represents an obstacle to TB control, especially in areas where MDRTB prevalence is high (*1*). New World Health Organization (WHO) estimates suggest that 424,203 MDRTB cases occurred in 2004 (95% confidence interval 376,019–620,061), or 4.3% of all new and previously treated TB cases. More than half of the estimated MDRTB cases were in China and India, while the highest estimated prevalences were in countries of the former Soviet Union and certain provinces of China (*2*).

DOTS is the internationally recommended strategy for TB control and is based on a 6-month treatment regimen with first-line drugs (isoniazid, rifampin, pyrazinamide, and ethambutol) for new patients and an 8-month treatment regimen with isoniazid, rifampin, pyrazinamide, ethambutol, and streptomycin for re-treatment patients (*3*). While DOTS prevents the emergence of drug resistance in drug-susceptible cases, in patients with MDRTB, this treatment yields inadequate cure rates (*4–7*). A retrospective cohort study of treatment of MDRTB with this regimen in 6 countries showed treatment success rates of 52% (range 11%–60%) in new cases and 29% (range 18%–36%) in previously treated cases (*5*). In addition, the frequency of TB recurrence among MDRTB patients previously considered to be cured after this treatment has been reported at 28% (*6*). Treating MDRTB with second-line drugs may cure >65% of patients and stop ongoing transmission (*8–10*). However, most of the evidence of successful MDRTB management is generated from high-income countries where treatment is provided in referral hospitals (*10*).

In 1999, WHO and partner agencies launched DOTS-Plus to manage MDRTB in resource-limited settings, a term that was recently abolished since it was used for the piloting of the management of MDRTB within the context of DOTS programs. Effective MDRTB control builds on

*World Health Organization, Geneva, Switzerland; †KNCV Tuberculosis Foundation, The Hague, the Netherlands; ‡Partners In Health, Boston, Massachusetts, USA; §Socios En Salud, Lima, Peru; ¶International Union Against Tuberculosis and Lung Disease, Paris, France; #Centers for Disease Control and Prevention, Atlanta, Georgia, USA; **Tartu University Clinics, Tartu, Estonia; ††National TB Programme, Tallinn, Estonia; ‡‡State Centre of Tuberculosis and Lung Diseases of Latvia, Riga, Latvia; §§Harvard Medical School, Boston, Massachusetts, USA; ¶¶MDR-TB Project in Tomsk Oblast, Tomsk, Russian Federation; and ##Makati Medical Center, Makati, the Philippines

the 5 tenets of the DOTS strategy (*3*) and expands each of these areas to address the complexities associated with treating MDRTB (*11*). As part of this strategy, a novel partnership known as the Green Light Committee (GLC) was created to foster access to, and rational use of, second-line drugs (*11–13*). The second-line drugs included in the WHO Model List of Essential Medicines are amikacin, capreomycin, ciprofloxacin, cycloserine, ethionamide, kanamycin, levofloxacin, ofloxacin, *p*-aminosalicylic acid, and prothionamide (*11*). GLC reviews applications from projects that wish to integrate MDRTB management into a DOTS-based TB control program. If the applicant proposes a strategy consistent with international recommendations and agrees to the monitoring procedures of GLC, then access to reduced-price, quality-assured second-line drugs is granted. Some of the requirements for GLC endorsement include a well-functioning DOTS program, long-term political commitment, rational case-finding strategies, diagnosis of MDRTB through quality-assured culture and drug susceptibility testing (DST), treatment strategies that use second-line drugs under proper management conditions, uninterrupted supply of quality-assured second-line drugs, and a recording and reporting system designed for MDRTB control programs that enables monitoring and evaluation of program performance and treatment outcome (*11*,*13*,*14*). These conditions represent the MDRTB control framework. Projects must be tailored to site-specific epidemiologic and programmatic conditions within this framework. As a result, MDRTB control programs may differ substantially between settings (*11*). Some aspects in which MDRTB control programs may vary include whether all TB patients are tested with culture and DST or only patients with an increased risk for MDRTB, use of standardized or individualized second-line treatment regimen, and hospitalization of MDRTB patients or provision of treatment on an ambulatory basis. This analysis of the first 5 GLC-endorsed MDRTB control programs provides, for the first time, results on management of MDRTB under DOTS-based program conditions in multiple resource-limited countries by using standardized treatment outcome definitions.

Methods

This is a study of MDRTB patients enrolled in Estonia, Latvia, Lima (Peru), Manila (the Philippines), and Tomsk Oblast (Russian Federation). The data were collected prospectively. The enrollment period started in 1999 for Lima and Manila, 2000 for Latvia and Tomsk, and 2001 for Estonia and ended December 31, 2001. All patients evaluated were managed under GLC-approved protocols and had the opportunity to receive ≥24 months of treatment. In addition, follow-up data on successfully treated patients were collected at the beginning of 2006, two years after the last patient's treatment ended (December 31, 2003).

A new MDRTB patient was defined as a patient who had never received TB treatment or who had received TB treatment for <1 month. An MDRTB patient previously treated with only first-line drugs was defined as an MDRTB patient who had been treated for >1 month with only first-line anti-TB drugs. An MDRTB patient previously treated with second-line drugs was defined as an MDRTB patient who had been treated for >1 month with ≥1 second-line anti-TB drug (with or without first-line drugs). Six standard and mutually exclusive categories were used to define treatment outcome: cure, treatment completed, death, default, failure, and transfer out (*14*) (Table 1). The treatment success percentage was obtained by adding the percentage of cured patients to the percentage of patients who completed treatment.

Outcome data were recorded by the individual projects in centralized electronic registers. International standards for core data collection in MDRTB control programs were developed in 2000 (*11*). Projects developed their own standardized forms and electronic databases that included all of the core data elements. Aggregated program and patient data were collected from each project with a data collection form developed by GLC. The accuracy of laboratory methods was verified though regular quality assurance exercises performed by a network of WHO/International Union Against Tuberculosis and Lung Disease supranational TB reference laboratories, as previously described (*1*). For each project, data submitted to WHO were

Table 1. Treatment outcome definitions for multidrug-resistant tuberculosis (MDRTB) patients (*14*)

Category	Definition
Cure	Completed treatment according to country protocol and been consistently culture-negative (≥5 results) for final 12 mo of treatment. If only 1 positive culture is reported during that time with no concomitant clinical evidence of deterioration, patient may still be considered cured, provided that positive culture is followed by >3 consecutive negative cultures taken ≥30 d apart.
Treatment completed	Completed treatment according to country protocol but does not meet definition for cure or treatment failure because of lack of bacteriologic results (i.e., ≤5 cultures were performed in the final 12 mo of therapy).
Death	Died for any reason during the course of MDRTB treatment.
Treatment default	Treatment was interrupted for ≥2 consecutive months for any reason.
Treatment failure	≥2 of 5 cultures recorded in the final 12 mo are positive or if any of the final 3 cultures is positive. Treatment will also be considered to have failed if a clinical decision has been made to terminate treatment early due to poor response or adverse events.
Transfer out	Transferred to another reporting and recording unit and the treatment outcome is unknown.

checked for completeness and consistency; all errors or discrepancies were corrected in consultation with the project's investigators.

Statistical tests were performed with the Fisher exact test for 2×2 comparisons and the χ^2 test for the other tables. For all statistical tests, we regarded a p value <0.05 as significant. Data were analyzed in Stata version 8 (StataCorp LP, College Station, TX, USA).

Results

The 5 programs are described in Table 2. All projects are conducted in well-established DOTS programs. Four projects are integrated into the national TB program (NTP): Estonia, Latvia, Lima, and Tomsk. The project in Manila is conducted by a nongovernmental organization at a private tertiary hospital, in close collaboration with NTP. All projects provide free care to MDRTB patients. The programs in Estonia, Latvia, and Tomsk are the only available treatment options for MDRTB, while in Lima and Manila, treatment in the private sector is also available.

In all projects, financing is obtained through both national healthcare budgets and external sources. All projects work in collaboration with technical agencies and, in Lima and Tomsk, nongovernmental organizations. Directly observed treatment (DOT) is standard care in all projects. Treatment is observed by a range of persons, including healthcare workers (primarily nurses) and community volunteers. DOT worker incentives are provided in Estonia, Lima, and Tomsk, primarily consisting of money (Estonia) and food and money (Lima and Tomsk). Patient incentives, food and free transportation, are provided in all projects except for those in Manila. In Lima, patients also receive housing and social, educational, and financial support, as needed. Lima and Manila offer patient support groups. Sputum culture and DST to first- and second-line drugs are performed at each project site except for Lima and Tomsk, which rely on an international laboratory for DST to second-line drugs. All projects test for susceptibility to several first- and second-line drugs. In 3 projects, those in Estonia, Latvia, and Tomsk, MDRTB patients are hospitalized in a separate ward or building until they are noninfectious. In Peru and Manila, only severely ill patients and patients with side effects are hospitalized.

The 5 projects use different case-finding strategies and treatment options (use of empiric treatment regimens while awaiting DST results or not) (Table 2). In Estonia, Latvia, and Tomsk, all (new and previously treated) patients received DST at the start of treatment. However, in this study MDRTB patients from Tomsk were all previously treated patients on a waiting list for treatment. In Lima, DST is only performed on isolates from patients in whom treatment failed or suspicion of MDRTB is high. Most patients in Lima were referred to the GLC-approved MDRTB control program only after failure of a standardized regimen, which contained second-line drugs and was

Table 2. Description of MDRTB control programs*

Factor	Estonia	Latvia	Lima	Manila	Tomsk
Start of enrollment	1 Aug 2001	1 Jan 2001	1 Feb 1999	15 Apr 1999	12 Sep 2000
Project size	Country	Country	Region	District	Region
Project population	1,364,101	2,350,000	7,748,258	9,930,000	1,032,400
Prisons included?	Yes	Yes	Yes (1 prison)	No	Yes
% MDRTB, new cases (2002)	11.9	9.8	NA†	NA†	13.7
% MDRTB, previously treated cases (2002)	29.3	26.7	NA†	NA†	43.6
Voluntary HIV counseling/testing?	Yes	Yes	Yes	No	Yes
Empiric regimen?‡	No	Yes	Yes	No	Yes
Surgery used?	Yes	Yes	Yes	No	Yes
DOT (days per wk)	7 hosp, 6 amb	7 hosp, 5–6 amb	6	6	6
Incentives to patients?	Yes	Yes	Yes	No	Yes
Incentives to providers?	Yes	No	Yes	No	Yes
Culture monitoring	Monthly	Monthly	Monthly	Monthly	Monthly
X-ray monitoring	Every 3 mo	Every 3 mo	Every 6 mo	Every 6 mo	Every 3 mo
Drugs for which DST is performed§	H, R, E, S, Z, Amk, Cm, Eth, Km, Ofx, Pth	H, R, E, S, Z, Cm, Cs, Eth, Km, Ofx, Pas, T	H, R, E, S, Z, Amk, Cfx, Cm, Cs, Eth, Km	H, R, E, S, Z, Amc,§ Amk, Km, Cfx, Clr,§ Lfx, Ofx	H, R, E, S, Z, Cm, Cs, Km, Ofx, Pas, Pth

*MDRTB, multidrug-resistant tuberculosis; NA, not applicable; DOT, directly observed treatment; hosp, hospital; amb, ambulatory; DST, drug susceptibility testing; H, isoniazid; R, rifampin; E, ethambutol; S, streptomycin; Z, pyrazinamide; Amk, amikacin; Cm, capreomycin; Eth, ethionamide; Km, kanamycin; Ofx, ofloxacin; Pth, prothionamide; Cs, cycloserine; Pas, p-aminosalicylic acid; T, thiacetazone; Cfx, ciprofloxacin; Amc, amoxicillin–clavulanic acid; Clr, clarithromycin; Lfx, levofloxacin.

†Lima and Manila do not perform routine drug resistance surveillance.

‡Use of treatment regimens based on the history of drugs used by the patient while awaiting DST results.

§Amc and Clr and are not included on the World Health Organization Model List of Essential Drugs and are therefore not purchased through the Green Light Committee. However, each project used additional drugs at its discretion.

used by the Peruvian NTP. In Manila, patients had a range of treatment histories; most came after failure of treatment provided by private physicians outside the DOTS program. Because of the long turnaround times for DST results from the international laboratory, patients in Lima and Tomsk often received empiric treatment after culture conversion. For each program, the drugs against which the strains were tested are given in Table 2; however, not all strains were tested against all the drugs listed for each program.

All projects used DST results and previous treatment history to design the individualized regimen. Across the 5 projects, regimens contained ≥4 drugs, and most patients received >4 drugs initially. All regimens included an injectable agent (amikacin, capreomycin, kanamycin, or streptomycin) and a fluoroquinolone (ciprofloxacin, levofloxacin, or ofloxacin). Nearly all drugs were administered for the duration of treatment except for the injectable agent, which was given for a specified interval after the patient's specimens were culture negative. Treatment duration was 18–24 months, and the exact length was usually determined individually for each patient. The frequency of drugs used in the regimens is shown in Table 3. The median duration of patient follow-up after a patient's having been declared cured or treatment completed was 24 months (range 12 months [Lima and Tomsk] to 36 months [Estonia]).

Drugs were administered under direct observation. In Lima, Tomsk, and Manila, drugs were administered 6 days per week; in Estonia and Latvia, drugs were given 7 days during the hospital phase and then 5 or 6 days a week after discharge. Monitoring of treatment regimens was based on the results of monthly sputum smear and culture. Chest radiographs were also performed every 3 months in Estonia, Latvia, and Tomsk and every 6 months in Lima and Manila. All projects except that in Manila had access to adjunctive surgery for major interventions such as lung resection. Each project provided patients with ancillary drugs to manage adverse events.

MDRTB program cohort characteristics are shown in Table 4. Among 1,047 MDRTB patients, 119 (11%) were new, and 928 (89%) were previously treated. Among the 919 previously treated patients from whom details could be obtained, 438 (48%) had received only first-line drugs and 481 (52%) first and second-line drugs. Few patients' isolates were resistant to only rifampin and isoniazid (2.6%); most (65%) were resistant to first- and second-line drugs. HIV coinfection was identified in 0% (Estonia and Tomsk) and 4.5% (Latvia) of patients. (In Lima and Tomsk, all MDRTB patients were tested for HIV; in Estonia and Latvia, 67% and 90% of MDRTB patients were tested; and in Manila HIV testing was not performed.) Frequency of hospitalization varied from 5.0% (Manila) to 100% (Latvia), and duration of hospitalization ranged from 29 days (Manila) to 267 days (Tomsk).

The treatment outcomes of new, previously treated, and all MDRTB patients are shown in Table 5 and Figure 1. Treatment was successful in 70% of 1,047 patients (range 59%–83%). Failure occurred in 3.3% to 11% of patients, default in 6.3% to 16%, and death in 3.7% to 19%. In Estonia and Latvia, MDRTB patients not previously treated for TB had a higher treatment success rate (80% vs. 61%, odds ratio [OR] 2.54, 95% confidence interval [CI] 1.47–4.37, $p<0.005$) and a lower failure rate (4.4% vs. 15%, OR 0.26, 95% CI 0.10–0.67, $p<0.005$) than previously treated patients. Adverse events led to treatment cessation in 3.2% of patients (range 0% [Tomsk] to 8.6% [Manila]). By the end of 2005, a total of 14 of 670 patients (2.1%) who were followed-up after cure or treatment completion had relapsed (range 1.1% [Lima] to 10.0% [Estonia]) (Table 6).

Table 3. Frequency of drugs used in multidrug-resistant tuberculosis control program treatment regimens

Drug	Estonia, n (%)	Latvia, n (%)	Lima, n (%)	Manila, n (%)	Tomsk, n (%)	Total, n (%)
Ethambutol	44 (95.7)	117 (47.8)	102 (20.1)	43 (41.0)	28 (19.6)	334 (31.9)
Pyrazinamide	1 (2.2)	99 (40.4)	146 (28.7)	88 (83.8)	84 (58.7)	418 (39.9)
Streptomycin	1 (2.2)	9 (3.7)	104 (20.5)	51 (48.6)	0	165 (15.8)
Capreomycin	11 (23.9)	115 (46.9)	199 (39.2)	23 (21.9)	94 (65.7)	442 (42.2)
Cycloserine	45 (97.8)	189 (77.1)	316 (62.2)	100 (95.2)	142 (99.3)	792 (75.6)
Ciprofloxacin	0	0	257 (50.6)	18 (17.1)	0	275 (26.3)
Clofazimine	0	0	13 (2.6)	0	0	13 (1.2)
Kanamycin	7 (15.2)	129 (52.7)	167 (32.9)	91 (86.7)	47 (32.9)	441 (42.1)
Levofloxacin	1 (2.2)	0	0	30 (28.6)	0	31 (3.0)
Ofloxacin	35 (76.1)	242 (98.8)	44 (8.7)	87 (82.9)	142 (99.3)	550 (52.5)
p-Aminosalicylic acid	26 (56.5)	71 (29.0)	323 (63.6)	98 (93.3)	118 (82.5)	636 (60.7)
Prothionamide or ethionamide	38 (82.6)	154 (62.9)	244 (48.0)	104 (99.0)	94 (65.7)	634 (60.6)
Augmentin	4 (8.7)	7 (2.9)	325 (64.0)	0	2 (1.4)	338 (32.3)
Clarithromycin	4 (8.7)	1 (0.4)	67 (13.2)	46 (43.8)	3 (2.1)	121 (11.6)
Sparfloxacin	0	0	0	14 (13.3)	0	14 (1.3)
Thiacetazone	0	164 (66.9)	0	0	0	164 (15.7)

Table 4. Multidrug-resistant tuberculosis control program cohort characteristics*

Characteristic	Estonia, n (%)	Latvia, n (%)	Lima, n (%)	Manila, n (%)	Tomsk, n (%)	Total, n (%)
Total no. cases	46 (100.0)	245 (100.0)	508 (100.0)	105 (100.0)	143 (100.0)	1,047 (100.0)
New cases	22 (47.8)	91 (37.1)	1 (0.2)	5 (4.8)	0	119 (11.4)
Previously treated cases	24 (52.2)	154 (62.9)	507 (99.8)	100 (95.2)	143 (100.0)	928 (88.6)
Cases previously treated with first-line drugs	19 (79.2)	132 (85.7)	125 (25.0)†	53 (54.6)†	109 (76.2)	438 (47.7)
Cases previously treated with first- and second-line drugs	5 (20.8)	22 (14.3)	376 (75.0)†	44 (45.4)†	34 (23.8)	481 (52.3)
Resistance to only H and R	0	7 (2.9)	11 (2.2)	9 (8.6)	0	27 (2.6)
Resistance to only H, R, and other first-line drugs	0	78 (31.8)	182 (35.8)	26 (24.8)	55 (38.5)	341 (32.6)
Resistance to first- and second-line drugs	46 (100.0)	160 (65.3)	315 (62.0)	70 (66.7)	88 (61.5)	679 (64.9)
Treatment cessation because of adverse events	3 (6.5)	5 (2.0)	NA	9 (8.6)	0	17 (3.2)
HIV coinfection	0	11 (4.5)	5 (1.0)	NA	0	16 (1.7)
Surgery performed	1 (2.2)	18 (7.3)	78 (15.4)	0	17 (11.9)	114 (10.9)
No. patients hospitalized	41 (89.1)	245 (100.0)	NA	5 (5.0)	71 (49.7)	362 (67.2)
Average no. drugs in treatment regimen	5.4	5.5	NA	6.28	5.3	

*H, isoniazid; R, rifampin; NA, not applicable.
†Information is lacking from 6 patients (Lima) and 3 patients (Manila) on previous treatment with first-line drugs only or with first- and second-line drugs.

Discussion

Today, management of MDRTB is included as a recommended part of the new Stop TB Strategy (*15*). WHO's guidelines have also been revised to encourage countries to collect drug resistance surveillance data from patients in different retreatment categories and to build capacity to diagnose and treat MDRTB within the context of DOTS (*16*). However, few NTPs in resource-limited settings have integrated effective treatment strategies for resistant cases (*17*).

The major perceived barrier to MDRTB treatment is the high cost of quality-assured second-line drugs. Additional

Table 5. Treatment outcomes of new, previously treated, and all multidrug-resistant tuberculosis patients

Patients	Estonia, n (%)	Latvia, n (%)	Lima, n (%)	Manila, n (%)	Tomsk, n (%)	Total, n (%)
New patients						
Cured	16 (72.7)	72 (79.1)	0	1 (20.0)	0	89 (74.8)
Completed	1 (4.5)	1 (1.1)	0	1 (20.0)	0	3 (2.5)
Default	3 (13.6)	13 (14.3)	0	2 (40.0)	0	18 (15.1)
Failed	1 (4.5)	4 (4.4)	0	0	0	5 (4.2)
Died	1 (4.5)	1 (1.1)	1 (100.0)	1 (20.0)	0	4 (3.4)
Transferred	0	0	0	0	0	0
Total	22 (100.0)	91 (100.0)	1 (100.0)	5 (100.0)	0	119 (100.0)
Treatment success	17 (77.3)	73 (80.2)	0	2 (40.0)	0	92 (77.3)
Previously treated patients						
Cured	12 (50.0)	93 (60.4)	351 (69.2)	60 (60.0)	118 (82.5)	634 (68.3)
Completed	1 (4.2)	2 (1.3)	0	0	0	3 (0.3)
Default	4 (16.7)	27 (17.5)	40 (7.9)	12 (12.0)	9 (6.3)	92 (9.9)
Failed	3 (12.5)	24 (15.6)	17 (3.4)	12 (12.0)	9 (6.3)	65 (7.0)
Died	4 (16.7)	8 (5.2)	98 (19.3)	15 (15.0)	7 (4.9)	132 (14.2)
Transferred	0	0	1 (0.2)	1 (1.0)	0	2 (0.2)
Total	24 (100.0)	154 (100.0)	507 (100.0)	100 (100.0)	143 (100.0)	928 (100.0)
Treatment success	13 (54.2)	95 (61.7)	351 (69.2)	60 (60.0)	118 (82.5)	637 (68.6)
All patients						
Cured	28 (60.9)	165 (67.3)	351 (69.1)	61 (58.2)	118 (82.5)	723 (69.1)
Completed	2 (4.3)	3 (1.2)	0	1 (1.0)	0	6 (0.6)
Default	7 (15.2)	40 (16.3)	40 (7.9)	14 (13.3)	9 (6.3)	110 (10.5)
Failed	4 (8.6)	28 (11.4)	17 (3.3)	12 (11.4)	9 (6.3)	70 (6.7)
Died	5 (10.9)	9 (3.7)	99 (19.5)	16 (15.2)	7 (4.9)	136 (13.0)
Transferred	0	0	1 (0.2)	1 (1.0)	0	2 (0.2)
Total	46 (100.0)	245 (100.0)	508 (100.0)	105 (100.0)	143 (100.0)	1047 (100.0)
Treatment success	30 (65.2)	168 (68.6)	351 (69.1)	62 (59.0)	118 (82.5)	729 (69.6)

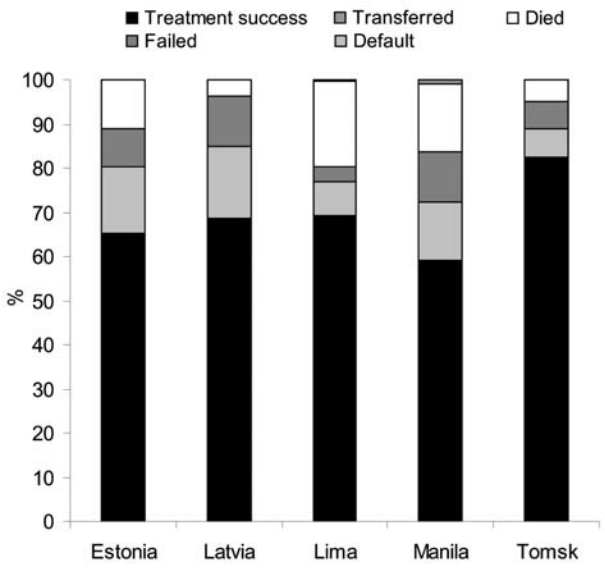

Figure 1. Treatment outcomes of multidrug-resistant tuberculosis patients in Estonia (46 patients), Latvia (245 patients), Lima (508 patients), Manila (105 patients), and Tomsk (143 patients).

barriers include extensive laboratory and monitoring requirements, adverse events associated with second-line drugs, low availability of quality-assured second-line drugs, difficulties in ensuring adequate patient support (including DOT) during the long treatment course, and the risk for resistance to second-line drugs (*18*,*19*). Consequently, many NTPs focus on achieving high cure rates in their DOTS programs and neither diagnose nor treat MDRTB (*17*).

This study represents the first multicountry evaluation of MDRTB patients treated in resource-limited settings under the GLC mechanism and endorsed by the respective NTP of each country. Although program design and patient management varied, the results show that treating MDRTB in resource-limited settings is feasible and effective. Treatment with second-line drugs is more successful than a 6- to 8-month regimen of first-line drugs for such patients and, in spite of a patient population characterized by high proportions of severe chronic cases with extensive resistance patterns, treatment outcomes of these projects match the outcomes of treatment with second-line drugs in wealthier settings (*10*). However, in each project, extensive training on managerial, laboratory, clinical, and social aspects of MDRTB control took place before GLC approval and initiation of treatment. Socioeconomic support was provided to the patients in 4 of the 5 sites, and in all sites a patient-centered approach was used for treatment delivery, with DOT ensured during the full course of treatment. These efforts may partly explain why the relapse rates were low (2.1%) and suggest such best practices are essential for a successful outcome. In addition, all projects were supported by technical agencies, and some benefited from extensive NGO support.

Significant differences were seen in favorable (cure and completed) and unfavorable (default, failure, died, and transferred out) outcomes between projects ($p = 0.002$), and although patient populations cannot be compared between projects as a result of different TB epidemiologic features in different countries, some general observations can be made with respect to the differences in treatment outcomes. Default rates were higher in Estonia, Latvia, and Manila than in Lima and Tomsk. TB specialists in Estonia and Latvia attributed the high default rates to a high proportion of patients with severe alcohol abuse disorders for whom adherence to treatment is difficult. A recent study in Latvia could not confirm that alcohol misuse was clearly linked to default, but the number of nonadherent patients was small and the statistical power correspondingly weak (*9*). Although the project in Tomsk also experienced problems with alcoholism, default rates were low because a large proportion of patients were imprisoned (41%) during the treatment period. The high default rate in Manila appeared to be related to the facts that at the beginning of Manila's project, treatment was delivered in a single site that was not easily accessible to all patients and that drugs to manage adverse reactions had to be purchased by the patients. In addition, during the reporting period, the program in Manila did not provide any patient or DOT worker incentives. The low default

Table 6. Clinical and bacteriologic progress after cure or treatment completion and number of relapses identified by the beginning of 2006

Characteristic	Estonia	Latvia	Lima	Manila	Tomsk	Total
Duration of follow-up after cure or treatment completion (mo)	36	24	12	24	12	
Frequency of follow-up after cure or treatment completion	Every 6 mo	Every 6 mo	Every 3 mo	Every 6 mo	Every 6 mo	
No. cured or completed treatment	30	168	351	62	118	729
No. followed up*	30	168	351	62	59	670
No. cured or completed treatment who relapsed by 2006	3	5	4	1	1	14
Relapse rate (95% confidence interval)	10.0% (2.11–26.53)	3.0% (0.97–6.81)	1.1% (0.31–2.89)	1.6% (0.04–8.66)	1.7% (0.04–9.09)	2.1% (1.14–3.51)

*In Tomsk, 59 patients left the region after having been declared cured. These patients were lost to follow-up.

rates in Lima and Tomsk could be attributed to a large variety of treatment delivery options and incentive and enabler programs for patients.

The high frequency of death in Lima likely reflects the fact that in a high proportion of patients, a standard MDRTB treatment regimen with second-line drugs was unsuccessful (*20*). The proportion of patients previously treated with second-line drugs was much higher in Lima (75%) than in other projects (14%–45%) (Figure 2). However, the proportions of patients with infections resistant to first- and second-line drugs were similar in Latvia, Lima, Manila, and Tomsk ($p = 0.47$). In Estonia, resistance patterns to first- and second-line drugs differed substantially when compared with patterns in the other 4 projects ($p<0.0001$), and in Estonia all patients had infections resistant to first- and second-line drugs (Table 4).

During the study period, only Estonia and Latvia routinely attempted to identify MDRTB patients at the start of their first treatment for TB, and the results show that early identification and referral may reduce death and treatment failure and thus improve treatment success. This finding is consistent with those of Turett et al. (*21*). The delay in the diagnosis of MDRTB results in treatment of patients with chronic disease, progressive parenchymal destruction, higher bacillary loads, and continuing transmission (*22*).

The study confirms that adverse events are manageable in the treatment of MDRTB in resource-limited settings. Few patients stopped treatment because of adverse events, which is similar to a previous report. Each project, however, applied intensive approaches to manage adverse events, including altering dosages when appropriate, administering ancillary drugs to treat adverse events, and discontinuing drugs. In addition, all projects conducted special training on adverse events to second-line drugs and used standard protocols for their registration (*23*).

Studies of the cost and cost-effectiveness of MDRTB management have been completed in Estonia (unpub. data), Manila (*24*), and Tomsk (*25*). From the health system perspective, the average cost per patient treated was approximately US $3,400 in the Philippines and US $9,000–$10,000 in Estonia and Tomsk. The higher costs in Estonia and Tomsk reflect considerable hospitalization during treatment (30%–50% of overall costs compared to 3% in the Philippines). The second-line drug costs ranged from US $1,600 in the Philippines to US $3,700 in Tomsk; second-line drugs were the highest cost items in the Philippines and Tomsk and the second highest in Estonia.

Our study has several limitations. First, risk factors for poor treatment outcomes could not be examined because data were in an aggregate form, not as individual patient data. The second limitation is that the results are not representative of all GLC-approved projects currently functioning. As mentioned, GLC projects are tailored to the local

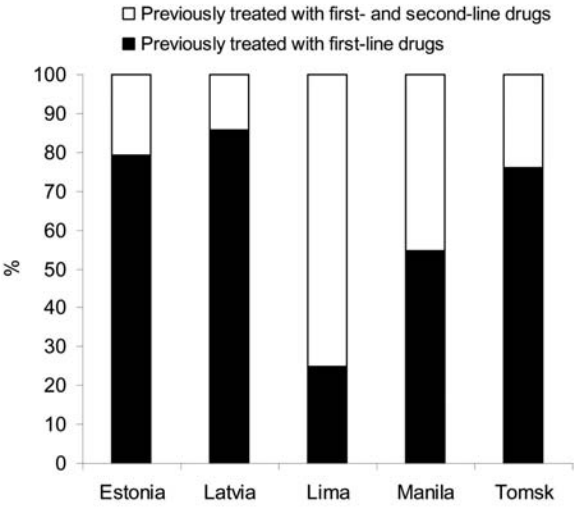

Figure 2. Proportion of multidrug-resistant tuberculosis patients in the 5 sites previously treated with first-line drugs only or with first- and second-line drugs.

health infrastructure, human and financial resources, and the epidemiologic situation. As a result, costs and outcomes differ between projects. Several projects have been approved by GLC that use standardized treatment regimens based on representative drug resistance surveillance data in relevant patient categories. In settings without a history of second-line drug use, MDRTB control is likely to yield better treatment outcome results. In these settings, susceptibility to the most effective second-line drugs may be preserved, permitting perhaps shorter regimens with fewer, less toxic drugs. As all GLC-approved MDRTB control projects record the same core data, information on success within each of the different approaches will be available within the next 3 years.

Conclusion

After successful piloting of MDRTB management within TB control programs, WHO and partners have reached the phase of expanding MDRTB control as a component of a comprehensive TB control program, which is described in the WHO guidelines for the treatment of TB (*3*), the new Stop TB Strategy (*15*), and in the new WHO guidelines for the programmatic management of drug-resistant tuberculosis (*26*). As countries are purchasing and using second-line drugs, the likelihood of misuse and creation of strains of TB resistant to all known anti-TB drugs increases. The GLC mechanism offers a way to provide access to care while ensuring rational and effective use of drugs. Beginning in 2002, the Global Fund to Fight AIDS, Tuberculosis and Malaria (GFATM) mandated that requests for second-line drugs for managing MDRTB should go through GLC to prevent their misuse. The GLC

model has been proposed to improve access to malaria (*27*) and HIV/AIDS treatment (*28*,*29*). As of May 2006, a total of 41 MDRTB control projects in 37 countries were endorsed by GLC, and >21,000 MDRTB patients were approved for treatment. The number of GLC-approved MDRTB control programs is increasing rapidly, both as a result of more funding for TB control from the GFATM and mainstreaming of MDRTB management into general TB control efforts. However, with the estimated incidence of 424,203 MDRTB cases, most cases remain undiagnosed and untreated. Expanding projects and accelerating evidence gathering are necessary to further develop international policies. The future success of MDRTB management in resource-limited settings will depend on the ability of the donor community and technical agencies, as well as TB-endemic countries themselves, to expand and strengthen MDRTB control programs.

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Fluoroquinolones and Risk for Methicillin-resistant *Staphylococcus aureus*, Canada

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Receipt of fluoroquinolones was the predominant risk factor for *Clostridium difficile*–associated disease (CDAD) during an epidemic in Quebec, Canada. To determine the role of antimicrobial drugs in facilitating healthcare-associated methicillin-resistant *Staphylococcus aureus* (MRSA) colonization and infection and to compare this role with their effects on methicillin-susceptible *S. aureus* infection and CDAD, we conducted a retrospective cohort study of patients in a Quebec hospital. For 7,371 episodes of care, data were collected on risk factors, including receipt of antimicrobial drugs. Crude and adjusted hazard ratios (AHR) were calculated by Cox regression. Of 150 episodes of MRSA colonization and 23 of MRSA infection, fluoroquinolones were the only antimicrobials that increased risk for colonization (AHR 2.57, 95% confidence interval [CI] 1.84–3.60) and infection (AHR 2.49, 95% CI 1.02–6.07). Effect of antimicrobial drugs on MRSA colonization and infection was similar to effect on CDAD and should be considered when selecting antimicrobial drugs to treat common infections.

Staphylococcus aureus remains an important nosocomial pathogen because of its virulence and adapting resistance mechanisms (*1–3*). Methicillin-resistant *S. aureus* (MRSA) has become widespread in hospitals worldwide and is now causing outbreaks in communities as well (*2*,*4–6*). In the United States, almost two thirds of *S. aureus* isolates from patients in intensive care units are methicillin resistant (*6*). In Canada, MRSA prevalence varies geographically and is highest in Quebec (27%) (*7*). In the Centre Universitaire Hospitalier de Sherbrooke, prevalence of MRSA increased from 5% of isolates in 2001 to 16% in 2004. Risk factors for infection by methicillin-sensitive *S. aureus* (MSSA) and MRSA include hospitalization, longer stay in a hospital, stay in an intensive care unit (ICU), more concurrent illnesses, residence in a long-term care facility, catheterization (central access or other venous), diabetes mellitus, cancer, surgery, wounds, hemodialysis, and HIV infection (*1*,*8–16*). Antimicrobial drugs, especially β-lactams, fluoroquinolones, and macrolides, have been incriminated as potentially facilitating MSSA and MRSA infections (*9*,*15–19*), but this association remains controversial. During a large epidemic of *Clostridium difficile*–associated disease (CDAD) in the province of Quebec, receipt of fluoroquinolones emerged as the predominant risk factor for CDAD in a large cohort study of inpatients at the Centre Universitaire Hospitalier de Sherbrooke, a 683-bed tertiary-care hospital (*20*). The commonality of risk factors for CDAD and MRSA has been noted before (*15*). To determine the role of various antimicrobial drugs in favoring healthcare-associated MRSA colonization and infection, we examined the same cohort of patients to identify risk factors for these outcomes and for MSSA infection.

Methods

Records of all adult patients hospitalized at least once from January 2003 through June 2004 in 3 wards (internal medicine, family medicine, gastroenterology) and a random sample of 50% of patients in the general surgery ward were retrospectively reviewed (*20*). For each patient, records of all admissions during this period were examined. To deal with multiple hospitalizations and repeated exposures, we used episodes of care (EOC) as the unit for all analyses (*20*). When intervals between hospital admissions were ≤60 days, distinct hospitalizations were considered as a single EOC and duration of stay was the sum of each admission within that EOC. Hospitalizations >60 days apart were defined as separate EOCs. We excluded

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hospitalizations for which the primary reason for admission was *S. aureus* infection or during which *S. aureus* infection was documented within 72 hours of admission.

The following data were collected for each patient: sociodemographic information, discharge diagnoses and concurrent illnesses, pharmaceutical drugs given, and laboratory test results. Receipt of antimicrobial drugs while in the hospital was abstracted from computerized medical records. When possible, receipt of antimicrobial drugs as outpatient therapy in the 2 months before the EOC was abstracted from the admission note. The overall amount of concurrent illness was measured according to the Charlson index (*21*). A patient with any of the following was considered immunosuppressed: HIV infection, leukemia, lymphoma, neutropenia, organ transplant, or other indications for receiving immunosuppressive drugs or systemic corticosteroids for ≥1 month. Potential outcomes were identified through a review of a laboratory database of clinical specimens (infections with MSSA or MRSA) and of the hospital's computerized database (MRSA colonization). When MRSA infection or colonization or MSSA infection developed, data with regard to risk factors were collected from date of first admission up to the date of microbiologic and clinical diagnosis. A case of nosocomial MRSA or MSSA infection was defined by 1) a positive *S. aureus* culture from a site considered infected by the treating physicians and for which antimicrobial drug therapy active against the pathogen was initiated or surgical drainage was performed and 2) occurrence of the above during an EOC or within 60 days of date of last discharge after an EOC. Patients were considered to have MRSA colonization if MRSA was recovered in a surveillance sample or in a clinical sample and the patient had not received vancomycin, linezolid, or cotrimoxazole. Patients for whom MSSA was found in a specimen but for whom no antistaphylococcal drug had been prescribed and for whom no surgical drainage had been performed were considered to have MSSA colonization and were not analyzed further.

During the study period, hospital policy was to screen all new patients who had been hospitalized during the previous year for MRSA colonization, by swabbing anterior nares, perineum, and dermal lesions (if any). Screening was thereafter repeated if the patient had contact with another patient who had MRSA or was transferred to ICU. Weekly screening for 4 consecutive weeks after an outbreak was also performed for patients in involved wards. Barrier precautions were initiated for all patients with MRSA colonization or infection. For the analysis of MRSA colonization, we excluded patients colonized with MRSA at the time of admission and patients who had no follow-up swabs taken to detect MRSA colonization after admission. A patient who satisfied the following criteria was considered to have acquired MRSA colonization: 1) no evidence of colonization at the time of admission (screening with negative results or no screening), 2) a positive result for MRSA during a follow-up screening, and 3) no evidence of active infection as defined by the administration of a drug active against MRSA or surgical drainage.

Crude and adjusted hazard ratios (AHR) were measured by using Cox regression analysis. Day 0 corresponded to the date of first admission in an EOC. Data were censored when the patient died or when 60 days had passed since the date of last discharge within that EOC, whichever came first. Variables significantly associated with the outcome in univariate analyses were tested in Cox multivariate models built up sequentially, starting with the variable most strongly associated with the outcome and continuing until no other variable reached significance. When the final model was reached, each variable was dropped in turn to assess its effect by using the likelihood ratio test. We kept in the final models variables that significantly enhanced the fit at the $p \leq 0.05$ level. Interactions were sought between variables that were independently associated with the outcomes. The proportional hazards assumption was verified by comparing the Kaplan-Meier curve to the Cox predicted curve for a given variable and by assessing the parallel nature of curves in log-log plots.

Until April 2003, clinical specimens and swabs for MRSA detection were put on plates of blood agar and mannitol salt agar, and *S. aureus* was confirmed by bound-coagulase test (Pasteurex, Sanofi Diagnostics Pasteur Ltd., Surrey, UK). Isolates found to be oxacillin resistant (≤10 mm) or to have intermediate resistance to oxacillin (11–12 mm) on a Kirby-Bauer disk diffusion assay were further tested by Etest on Mueller-Hinton agar with 2% sodium chloride incubated for 24 hours at 37°C. Those with a MIC ≥4 μmol/mL were considered to be MRSA. Since April 2003, MRSA has been confirmed by identifying the *mecA* gene by using PCR (LightCycler, Roche, Mannheim, Germany) in addition to oxacillin Etest.

Results

Patient Characteristics

Of 7,421 EOCs in the original cohort (*20*), 50 were excluded because a staphylococcal infection was the primary reason for admission or was documented within 72 hours of admission, which left 7,371 EOCs for the analysis of MRSA and MSSA infections. Of these patients, 3,432 (47%) were male, median age was 72 years, only one fifth (21%) had no concurrent illness (Charlson score 0), 21% stayed in ICU, and 20% had surgery. Almost half (46%) received antimicrobial drugs, most commonly fluoroquinolones (22.9%), second-generation cephalosporins (13.6%), metronidazole (9.1%), and first- (8.5%) and third-generation cephalosporins (7.7%). A nosocomial

MRSA infection developed in 23 patients (8 respiratory tract, 6 surgical wound, 4 urinary tract, 2 endovascular, 2 osteomyelitis, and 1 mediastinitis), and a nosocomial MSSA infection developed in 66 (15 respiratory tract, 15 soft tissue, 14 surgical wound, 7 endovascular, 7 urinary tract, 5 osteomyelitis, 3 mediastinitis).

MRSA Colonization

For the analysis of risk factors for MRSA colonization, 2,767 EOCs were retained. The others were excluded because of colonization at time of admission (n = 84) or because no follow-up screening for MRSA was performed (n = 4,520). The proportion of patients who had ≥1 follow-up screening assay for MRSA colonization increased from 5.7% of those hospitalized for 1 to 3 days to 74.2% of patients hospitalized for >15 days. Compared with the larger cohort described above, patients in this smaller cohort were older (median age 75 years), more likely to have concurrent illness (12%), more likely to have stayed in ICU (31%), more likely to have had surgery (25%), and more likely to have received antimicrobial drugs (57%), specifically fluoroquinolones (31.4%), second-generation cephalosporins (17.6%), metronidazole (13.6%), first-generation cephalosporins (13.0%), and third-generation cephalosporins (10.8%).

MRSA colonization developed in 150 patients. After confounding variables were adjusted for, the independent risk factors were age, duration of hospitalization, peptic ulcer disease, and receipt of fluoroquinolones. Receipt of narrow-spectrum penicillins had a protective effect (Table 1). Sex and an immunosuppressed condition were not associated with MRSA colonization (data not shown). Although their 95% confidence intervals (CIs) encompassed the null value, the protective effect of cotrimoxazole and the deleterious effect of H2-blockers significantly enhanced the fit and were retained in the final model. The association between use of fluoroquinolones and colonization with MRSA was not modified by duration of treatment (data not shown) but was somewhat stronger for those who received ciprofloxacin (n = 576, AHR 2.53, 95% CI 1.73–3.69) than those who received levofloxacin (n = 167, AHR 1.77, 95% CI 0.95–3.28). When both drugs were given sequentially, the AHR was higher (n = 79, AHR 5.18, 95% CI 2.99–8.96). After adjustment for confounders, none of the other antimicrobial drugs was associated with MRSA colonization. Receipt of clindamycin tended to be associated with colonization with MRSA (AHR 1.87, 95% CI 0.93–3.74, p = 0.08), but it was given to only 2.6% of patients. No interaction was found.

MRSA Infection

For analysis of MRSA infections, we could use all 7,371 EOCs, but power was limited by the small number of outcomes (n = 23). MRSA colonization at time of admission was by far the strongest independent risk factor for MRSA infection (Table 2). The other independent risk factors were having undergone surgery, having received fluoroquinolones or systemic corticosteroids, and having a history of peptic ulcer disease. Sex and immunosuppression were not associated with MRSA infection (data not shown). For the fluoroquinolones (whose median duration of use was 5 days), AHR was higher for the 958 patients who received this class of antimicrobial drugs for ≥5 days (AHR 3.70, 95% CI 1.49–9.18) because MRSA infection did not develop in any of the 699 patients who received fluoroquinolones for 1 to 4 days. The association was stronger for those who received both ciprofloxacin and levofloxacin (n = 124, AHR 4.61, 95% CI 1.16–18.41), intermediate for those who received only ciprofloxacin (n = 1124, AHR 2.25, 95% CI 0.84–6.01), and absent for those who received only levofloxacin (n = 363, AHR 1.10, 95% CI 0.13–9.35). None of the other classes of antimicrobial drugs was associated with MRSA infection after adjustment for confounders. According to multivariate analysis, the following were no longer associated with MRSA infection: age, duration of hospital stay, a high Charlson score, peripheral vascular disease or ischemic heart disease, ICU stay, and tube feeding. No interaction was found. The small number of outcomes precluded the identification of factors protective against MRSA infection.

MSSA Infection

For MSSA infections (Table 3), the independent risk factors were procedures and level of care (surgery, ICU stay, enteral feeding) and some specific medical conditions (diabetes mellitus, chronic renal failure, peripheral vascular disease). Univariate analyses showed several classes of antimicrobial drugs to be associated with MSSA, but none remained significant after adjustment for confounders. Sex and immunosuppression were not associated with MSSA infection (data not shown). No interaction was found.

Discussion

Interpretation of studies of the association of antimicrobial drug use and MRSA colonization or infection have been plagued by methodologic problems such as case-control design (prone to biases in the selection of controls), lack of adjustment for confounding variables, and the use of cases of MSSA colonization or infection as controls (in which instance an antimicrobial drug thought to be associated with MRSA can merely be protective against MSSA, or vice-versa) (*9*,*17*,*19*,*22*). For these reasons, the association between β-lactam antimicrobial drugs and MRSA colonization or infection remains unclear, in contrast with the more consistent relationship between these outcomes and use of fluoroquinolones (*9*,*17–19*,*22–24*). We avoided

Table 1. MRSA colonization according to demographic, clinical, and pharmaceutical characteristics during 2,767 episodes of care*

Characteristic	Crude hazard ratio (95% CI)	Adjusted hazard ratio (95% CI)
Age, y		
18–64	1.00	1.00
65–79	1.60 (0.96–2.68)	1.37 (0.82–2.30)
≥80	3.04 (1.90–4.86)†	2.66 (1.64–4.31)†
Hospital stay,‡ days		
1–7	1.00	1.00
8–14	2.24 (1.01–4.99)§	2.01 (0.90–4.49)
≥15	4.69 (2.28–9.63)†	3.22 (1.55–6.72)§
Charlson comorbidity index		
0	1.00	NS
1–3	1.46 (0.75–2.86)	
4–6	2.42 (1.24–4.73)§	
≥7	1.88 (0.89–4.00)	
History of		
Diabetes mellitus	1.13 (0.16–8.23)	NS
Chronic renal failure	1.52 (1.07–2.18)§	NS
Peripheral vascular disease	1.16 (0.83–1.63)	NS
Ischemic heart disease	1.34 (0.97–1.84)	NS
Peptic ulcer disease	1.89 (1.26–2.83)§	1.70 (1.13–2.54)§
Procedures and care		
ICU stay	1.19 (0.85–1.67)	NS
Surgery	1.09 (0.77–1.55)	NS
Tube feeding	1.63 (0.94–2.83)	NS
Antimicrobial drugs received		
Quinolones	2.88 (2.09–4.00)†	2.57 (1.84–3.60)†
Cephalosporins		
1st generation	0.87 (0.54–1.41)	NS
2nd generation	1.48 (1.02–2.14)§	NS
3rd generation	1.41 (0.90–2.22)	NS
Macrolides	1.11 (0.58–2.10)	NS
Clindamycin	2.23 (1.13–4.37)	NS
IV β-lactam/β-lactamase inhibitors	1.39 (0.84–2.31)	NS
Amoxicillin/clavulanic acid	1.25 (0.58–2.67)	NS
Carbapenems	1.67 (0.62–4.52)	NS
Narrow-spectrum penicillins¶	0.70 (0.39–1.26)	0.45 (0.24–0.85)§
Aminoglycosides	1.56 (0.87–2.85)	NS
Cotrimoxazole	0.62 (0.23–1.67)	0.27 (0.07–1.08)
Metronidazole	2.22 (1.55–3.20)†	NS
IV vancomycin	0.93 (0.41–2.10)	NS
Oral vancomycin	1.31 (0.56–3.09)	NS
Other drugs received		
Proton pump inhibitors	1.62 (1.15–2.27)§	NS
H2 blockers	1.43 (1.01–2.02)§	1.37 (0.94–1.96)
Corticosteroids	1.33 (0.95–1.88)	NS

*MRSA, methicillin-resistant *Staphylococcus aureus*. CI, confidence interval; NS, not significant; ICU, intensive care unit; IV, intravenous.
†p<0.001.
‡Duration of stay, including all admissions during that episode of care.
§p<0.05.
¶Penicillin, ampicillin, amoxicillin, cloxacillin.

some of these pitfalls by using a cohort design that had a sample large enough to allow adjustment for multiple confounding variables and in which patients with MRSA colonization or infection and MSSA infection were compared with those without such outcomes.

Fluoroquinolones were the only class of antimicrobial drugs associated with MRSA colonization and infection. AHRs were 2.57 and 2.49, respectively, presumably as a result of their disruption of the patient's complex microbiological flora, the selective inhibition of susceptible strains, and the increase in bacterial adhesion with surface fibronectin binding proteins after exposure to ciprofloxacin (*25*,*26*). Given the nearly identical AHRs for the association between fluoroquinolones and MRSA colonization and infection, the latter was not likely due to confounding by indication (e.g., clinicians who initiated

ciprofloxacin as empirical treatment for nosocomial infection ultimately found to be caused by MRSA). Cotrimoxazole tended to confer protection against MRSA, in agreement with a recent study showing that cotrimoxazole prophylaxis reduces the incidence of community-acquired MRSA among HIV-infected adults (*27*).

Table 2. MRSA infection according to demographic, clinical, and pharmaceutical characteristics during 7,371 episodes of care*

Characteristic	Crude hazard ratio (95% CI)	Adjusted hazard ratio (95% CI)
MRSA colonization at admission†		
Screening negative	1.00	1.00
No screening	1.63 (0.57–4.70)	1.69 (0.58–4.88)
Screening positive	53.46 (16.95–168.6)‡	43.66 (13.46–141.6)‡
Age, y		
18–64	1.00	NS
65–79	2.90 (1.04–8.08)§	
≥80	0.88 (0.24–3.29)	
Hospital stay,¶ days		
1–7	1.00	NS
8–14	1.34 (0.37–4.81)	
≥15	2.76 (1.00–7.65) §	
Charlson comorbidity index		
0	1.00	NS
1–3	2.15 (0.47–9.83)	
4–6	2.41 (0.48–11.98)	
≥7	5.27 (1.01–27.43)§	
History of		
Diabetes mellitus	1.62 (0.69–3.83)	NS
Chronic renal failure	1.14 (0.39–3.36)	NS
Peripheral vascular disease	2.60 (1.14–5.91)§	NS
Ischemic heart disease	2.47 (1.07–5.71)§	NS
Peptic ulcer disease	4.95 (2.10–11.69)‡	4.79 (1.99–11.53)‡
Procedures and care		
ICU stay	3.08 (1.35–7.03)§	NS
Surgery	4.62 (2.01–10.59)‡	5.70 (2.41–13.48)‡
Tube feeding	5.60 (1.88–16.67)§	NS
Antimicrobial drugs received		
Quinolones	4.65 (2.00–10.81)‡	2.49 (1.02–6.07)§
Cephalosporins		
1st generation	3.19 (1.25–8.15)§	NS
2nd generation	2.53 (1.04–6.17)§	NS
3rd generation	1.06 (0.25–4.54)	NS
Macrolides	0.82 (0.11–6.10)	NS
Clindamycin	4.37 (1.02–18.71)§	NS
IV β-lactam/β-lactam inhibitors	0.79 (0.11–5.91)	NS
Amoxicillin/clavulanic acid	1.80 (0.24–13.45)	NS
Carbapenems	0.00	NS
Narrow-spectrum penicillins#	2.25 (0.76–6.64)	NS
Aminoglycosides	2.20 (0.51–9.43)	NS
Cotrimoxazole	0.00	NS
Metronidazole	3.02 (1.18–7.72)§	NS
IV vancomycin	3.48 (0.81–15.02)	NS
Oral vancomycin	0.00	NS
Other drugs received		
Proton pump inhibitors	1.27 (0.54–3.01)	NS
H2 blockers	0.00	NS
Corticosteroids	3.06 (1.34–7.01)§	2.42 (1.02–5.75)§

*MRSA, methicillin-resistant *Staphylococcus aureus*; CI, confidence interval; NS, not significant; ICU, intensive care unit; IV, intravenous.
†MRSA screening includes swabbing of anterior nares, perineum, and dermal lesions.
‡p<0.001
§p<0.05
¶Duration of stay, including all admissions during that episode of care.
#Penicillin, ampicillin, amoxicillin, cloxacillin.

Aminoglycosides were not associated with CDAD (*20*), MSSA, or MRSA.

The effect of fluoroquinolones and cotrimoxazole on MRSA colonization and infection in patients was consistent with their activity in vitro. Of the 23 clinical isolates of MRSA from our patients, all were resistant to ciprofloxacin, and all were sensitive to cotrimoxazole. Among all clinical isolates of MRSA obtained during the study period, 96% (346/361) were resistant to ciprofloxacin and only 2% (8/361) were resistant to cotrimoxazole. For clinical isolates of MSSA, 6% (87/1,538) and 1% (10/1,538) were resistant to ciprofloxacin and cotrimoxazole, respectively.

Table 3. MSSA infection according to demographic, clinical, and pharmaceutical characteristics during 7,371 episodes of care*

Characteristic	Crude hazard ratio (95% CI)	Adjusted hazard ratio (95% CI)
Age, y		
18–64	1.00	1.00
65–79	0.69 (0.41–1.18)	0.56 (0.32–0.98)‡
≥80	0.29 (0.14–0.60)†	0.38 (0.18–0.83)‡
Hospital stay§, days		
1–7	1.00	
8–14	2.30 (1.21–4.38)‡	NS
≥15	1.74 (0.92–3.31)	
Charlson comorbidity index		
0	1.00	
1–3	1.10 (0.51–2.38)	
4–6	1.56 (0.70–3.50)	NS
≥7	3.58 (1.57–8.19)‡	
History of		
Diabetes mellitus	3.00 (1.85–4.86)†	2.24 (1.33–3.79)‡
Chronic renal failure	2.26 (1.33–3.85)‡	1.98 (1.11–3.55)‡
Peripheral vascular disease	1.92 (1.17–3.14)‡	1.73 (1.02–2.96)‡
Ischemic heart disease	1.37 (0.84–2.23)	NS
Peptic ulcer disease	0.92 (0.40–2.14)	NS
Procedures and care		
ICU stay	5.40 (3.27–8.93)†	3.16 (1.78–5.63)†
Surgery	6.45 (3.87–10.76)†	4.95 (2.83–8.66)†
Tube feeding	6.74 (3.68–12.35)†	2.19 (1.12–4.29)‡
Antimicrobial drugs received		
Quinolones	1.03 (0.59–1.78)	NS
Cephalosporins		
1st generation	2.29 (1.26–4.17)‡	NS
2nd generation	0.98 (0.50–1.93)	NS
3rd generation	1.07 (0.46–2.48)	NS
Macrolides	0.28 (0.04–2.00)	NS
Clindamycin	2.11 (0.66–6.73)	NS
IV β-lactam/β-lactam inhibitors	2.23 (1.05–4.73)‡	NS
Amoxicillin/clavulanic acid	1.15 (0.28–4.71)	NS
Carbapenems	4.19 (1.30–13.52)‡	NS
Narrow-spectrum penicillins¶	2.04 (1.06–3.92)‡	NS
Aminoglycosides	2.14 (0.92–4.98)	NS
Cotrimoxazole	0.42 (0.06–3.05)	NS
Metronidazole	1.26 (0.62–2.56)	NS
IV vancomycin	3.30 (1.40–7.75)‡	NS
Oral vancomycin	1.01 (0.24–4.17)	NS
Other drugs		
Proton pump inhibitors	1.31 (0.80–2.14)	NS
H2 blockers	4.31 (2.65–7.02)†	NS
Corticosteroids	1.56 (0.91–2.67)	NS

*MSSA, methicillin-sensitive *Staphylococcus aureus;* CI, confidence interval; NS, not significant; ICU, intensive care unit; IV, intravenous.
†p<0.001.
‡p<0.05.
§Duration of stay, including all admissions during that episode of care.
¶Penicillin, ampicillin, amoxicillin, cloxacillin.

Taken together, these findings emphasize the need to decrease the use of fluoroquinolones, which are given to almost one fourth of all inpatients. In Quebec, among subgroups of patients who do not have preexisting renal disease and who receive antimicrobial drugs to treat infections that are not life-threatening, the potential adverse consequences of aminoglycoside nephrotoxicity might be less than those of infections with MRSA and *C. difficile* triggered by fluoroquinolones. A more selective use of fluoroquinolones is possible, for instance, for patients with urinary tract infections caused by pathogens sensitive to cotrimoxazole or for patients who have intraabdominal infections for whom β-lactam/β-lactamase inhibitors might be considered. In contrast with its effect on MRSA, use of antimicrobial drugs had little effect on the risk for MSSA nosocomial infections. The risk factors identified were those reported in the literature (*1*,*12*) and offer little opportunity for prevention.

The association between peptic ulcer disease and both MRSA colonization and infection is intriguing but needs to be interpreted with caution, given that 19 specific medical conditions were tested. H2-blocker drugs were also associated with MRSA colonization, according to univariate analysis, and had borderline significance according to multivariate analysis. Whether drugs that change gastric pH might alter the microflora of the stomach and feces in a way that facilitates MRSA colonization deserves further study (*28*,*29*).

The major limitations of our study lie in its observational nature. Patients not hospitalized during the previous year were not screened for MRSA at the time of admission. We considered such patients to be noncolonized initially, and misclassification was unlikely, given the rarity of truly community-acquired MRSA in our region. Subsequent swabs to detect MRSA colonization were obtained from a selected subsample of patients, who might have differed from those not tested for characteristics related to the outcome. The surveillance system selected patients at somewhat higher risk for MRSA colonization or infection, if only because they were hospitalized longer. Whether our findings can be extrapolated to low-risk patients is unknown. The study was conducted in a hospital with ≈16% prevalence of methicillin resistance among isolates of *S. aureus*, which limited the number of outcomes, especially for MRSA infection.

In conclusion, in a tertiary-care hospital with an intermediate level of MRSA prevalence, fluoroquinolones were the only antimicrobial drugs associated with MRSA colonization and infection and, in conjunction with infection control measures, represented the pharmacologic risk factor most amenable to correction. Before the *C. difficile* epidemic in hospitals of Quebec, the use of fluoroquinolones had been very high, as in the United States (*30*). The risk of inducing MRSA and CDAD should be taken into consideration when selecting antimicrobial drugs to treat common infections.

Dr LeBlanc is a fellow in infectious diseases and clinical microbiology at the University of Sherbrooke, Canada. Her research interests include methicillin-resistant *Staphylococcus aureus*.

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Differentiation of Tuberculosis Strains in a Population with Mainly Beijing-family Strains

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A high prevalence of tuberculosis (TB) isolates that are genetically homogenous and from the Beijing family has been reported in Russia. To map TB transmission caused by these strains, new genotyping systems are needed. Mycobacterial interspersed repetitive units (MIRUs) offer the possibility of rapid PCR-based typing with comparable discrimination to IS*6110* restriction fragment length polymorphism techniques. Spoligotyping and detection of IS6110 insertion in the *dnaA-dnaN* region were used to identify Beijing strains in 187 *Mycobacterium tuberculosis* isolates from Samara, Russia. The Beijing isolates were analyzed by using 12-MIRU and 3–exact tandem repeats (ETR) loci and by an expanded set of 10 additional variable number tandem repeats loci. The expanded set of 25 MIRUs provided better discrimination than the original set of 15 (Hunter-Gaston diversity index 0.870 vs 0.625). Loci MIRU 26, 1982, and 3232 were the most polymorphic in Beijing isolates.

Rising rates of tuberculosis (TB) (*1*) and increasing resistance of drugs are substantial barriers to successful patient management and TB control programs. Russia, named by the World Health Organization as one of the 22 countries with the highest TB prevalence, has seen increasing rates of TB and HIV during the past decade (*2*–*6*) and high levels of resistance, particularly to multiple drugs (*7*,*8*).

The high discriminatory power of restriction fragment length polymorphism (RFLP) analysis based on the insertion sequence IS*6110* has provided the backbone of these analyses (*9*). Unfortunately, the technique is time-consuming, technically demanding, and insufficiently discriminating when used alone with isolates containing <5 IS*6110* sequences in the genome (*10*,*11*). Isolates with low numbers of copies account for as much as 20% of TB isolates in some populations (*12*).

Techniques based on PCR amplification of repetitive sequences are more rapid than RFLP, but their discriminatory power is usually lower. Among these techniques, spoligotyping is widely used to differentiate strains belonging to the *Mycobacterium tuberculosis* complex. Spoligotyping has been particularly useful for identifying strains belonging to the Beijing/W family of *M. tuberculosis* because of the characteristic spoligotyping pattern with the absence of spacers 1–34 in the direct repeat (DR) region of the *M. tuberculosis* genome (*13*). Beijing family strains are dominant across many Asian and former Soviet Union countries, and W strains are responsible for outbreaks of multidrug-resistant TB in the United States. These strains are now considered to be members of the same phylogenetic lineage, sharing key characteristics such as a similar RFLP pattern of 15–26 bands, IS*6110* insertions in the *dnaA-dnaN* and NTF-1 chromosomal regions, a characteristic pattern of single nucleotide polymorphism, and a spoligotyping pattern with the presence of spacers 35–43 and absence of spacers 1–34 in the DR region of the *M. tuberculosis* genome (*14*–*17*). Detecting the IS*6110* insertion in the *dnaA-dnaN* intergenic region may also identify the Beijing/W genotype (*14*,*15*).

Several studies have shown that a high proportion of TB isolates in Russia (particularly those that are drug resistant) belong to the Beijing family (*7*,*8*,*18*). To assess TB transmission in Russia, any genotyping system must be able to discriminate among Beijing strains. The identification of variable number tandem repeats (VNTRs) (*19*) in *M. tuberculosis* has offered the possibility of rapid ampli-

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fication-based techniques with comparable discrimination to RFLP-IS*6110* typing (*20–23*).

As with other PCR-based genotyping techniques, VNTR analysis uses small quantities of crude bacterial lysates; it is less labor-intensive than RFLP-IS*6110* typing, and automation is relatively straightforward (*22*). Moreover, determination of a limited number of polymorphic loci can provide sufficient discriminative power for a given local population and may increase the cost-effectiveness of molecular typing. Several panels of *M. tuberculosis* VNTRs have been used with some success previously: exact tandem repeats (ETRs) (*19*), MIRUs (*21*,*22*), and 2 panels of loci known as QUB and Mtub (*24–27*). Our aim was to determine the discriminative power of an expanded set of 25 VNTR loci when applied to TB strains in Russia where Beijing strains dominate.

Methods

M. tuberculosis Strains

A total of 187 *M. tuberculosis* strains were analyzed. They were selected from 880 *M. tuberculosis* strains isolated from patients (1 isolate per patient) with radiologically confirmed pulmonary TB, identified from all TB treatment facilities across Samara Oblast in central Russia (12 civilian TB hospitals and dispensaries and 1 prison TB hospital) during 2001–2002. The test panel included 138 (33.7%) of 409 strains isolated from patients in the civilian TB hospitals and dispensaries and 49 (10.4%) of 471 isolates from prisoners. Within sets of cultures isolated from civilians, every third isolate was selected for this study. Because isolates from prisoners were overrepresented, every 10th isolate was selected. The representation of isolates in the test panel was approximately proportional to the number of TB isolates from each clinical site. In the Samara region, the incidence of TB at the time of the study was 86.1/100,000 population.

Patient populations are described in previous publications (*18*,*28*). All patients with TB are tested for HIV as a part of routine practice in Russia. History of vaccination with *M. bovis* BCG was confirmed by presence of an appropriate scar.

Molecular Epidemiologic Analysis

Crude DNA extracts were obtained by heating cell suspensions with chloroform at 80°C as previously described (*29*). Spoligotyping used a standardized method (*30*) to identify isolates belonging to the Beijing family; results were confirmed by analysis of the *dnaA* to *dnaN* region in all isolates. The IS*6110* insertion in the origin of replication was detected between *dnaA* and *dnaN* genes as described previously (*15*,*16*). Briefly, PCR was performed in a 20-μL volume of 2 μL 10× PCR buffer (Bioline Ltd, London, UK); 0.5 units Taq polymerase (Bioline Ltd); 0.5 μL 2 mmol dNTP mixture (Bioline Ltd); 0.5 μL 20-μmol mix of forward and reverse primers, 15.5 μL water, and 1 μL of crude DNA extract. Thermal cycling was performed on a PerkinElmer 9700 thermocycler (PerkinElmer, Warrington, UK) as follows: 4 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 60°C, and 2 min at 72°C; followed by 7 min at 72°C and holding at 4°C. Amplification products were analyzed by electrophoresis on 1.5% agarose gel. Strains with insertion (i.e., Beijing family) yielded a product of ≈2 kb, and fragments of ≈550 bp (no insertion) indicated strains other than Beijing.

All 187 TB isolates were tested by using the set of 12-MIRU loci and the 3-ETR (A, B, and C) loci (*19*,*22*) (nos. 1–15, online Appendix Table, available from http://www.cdc.gov/ncidod/EID/vol12no09/04-1263_appT.htm). Beijing isolates were further analyzed by using an additional panel of VNTR loci (0424, 0531, 1955, 1982, 2074, 2163a, 3232, 3239, 3336, and 3690, online Appendix Table). Primers for loci at which predicted fragment size would exceed 1 kb were redesigned to enable analysis with the CEQ8000 equipment (Beckman Coulter, Fullerton, CA, USA).

Multiplex and simplex PCRs were performed, taking into consideration dye labeling and expected length of PCR products. Simplex PCR was used to amplify fragments in loci MIRU 20, ETR-C, MIRU 26, VNTR 424, VNTR 531, VNTR 1955, VNTR 1982, VNTR 2163a, VNTR 3232, VNTR 3239, and VNTR 3336. For other loci, multiplex PCR mixtures were prepared as follows: set 1 contained MIRU 4 and MIRU 16, set 2 contained MIRU 39 and ETR-A, set 3 contained MIRU 2 and MIRU 24, set 4 contained MIRU 31 and MIRU 40, set 5 contained MIRU 10 and MIRU 23, set 6 contained MIRU 27 and ETR-B, and set 7 contained VNTR 2074 and VNTR 3690.

For all mixtures, PCR was performed in 10-μL volumes containing 1 μL 10× PCR buffer (containing 1.5 mmol/L $MgCl_2$, Bioline Ltd); 0.5 U Taq polymerase (Bioline Ltd); 0.25 μl 2-mmol dNTP mixture (Bioline Ltd); 0.5 μL 20-μmol mixture of forward and reverse primers as described above, 7.0 μL water, and 1 μL DNA extract. For loci VNTR 424, VNTR 531, VNTR 1955, VNTR 1982, VNTR 2074, VNTR 2163a, VNTR 3232, VNTR 3239, VNTR 3336, and VNTR 3690, the mixture also contained 0.5 μL dimethylsulfoxide (Sigma, Dorset, UK). Thermal cycling programs were identical for all loci, and thermal cycling was performed on a PerkinElmer 9700 thermocycler by using the following parameters: 3 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 60°C, and 60 s at 72°C; followed by 5 min at 72°C.

Automated analysis of PCR fragments length was performed by using a Beckman Coulter CEQ8000 automatic sequencer. Multiple PCR products were analyzed in

capillaries as follows: capillary 1 contained PCR products for loci 2, 4, 10, 16, 23, and 24; capillary 2 for loci 27, 31, 39, ETR-A, and ETR-B; capillary 3 for loci 20, 26, and ETR-C; capillary 4 for loci 0424, 0531, and 1955; capillary 5 for loci 2974, 3232, 3239, and 3690; and capillary 6 for loci 1982, 2163a, and 3336.

Before being loaded onto the sequencer, PCR products were diluted in purified water (Sigma) as follows: products labeled with dye 2 were diluted to 10-fold; with dye 3, to 30-fold; and with dye 4, to 60-fold. A total of 1 μL of diluted PCR products mixture was added to 25 μL formamide (Beckman Coulter) containing 0.1 μL of DNA size standard 600 (Beckman Coulter), and 0.1 μL DNA size standard 640–1,000 (Bio Ventures Inc., Murfreesboro, TN, USA), labeled with dye 1. Fragment length estimation was performed by using proprietary software (Beckman Coulter).

Molecular weights of PCR-generated fragments for loci 1982 and 3232 for some isolates exceeded 1 kb, and results of automated fragment analysis were inconsistent. Molecular weights of PCR products and numbers of MIRU repeats for these strains were determined manually by electrophoresis on a 1.2% agarose gel (Agarose 1000, Invitrogen Ltd, Paisley, UK) with a 100-bp step DNA ladder as fragment size standard (Promega, Madison, WI, USA) (Figure).

Automated calling (in which PCR fragment sizes and allele assignment are automatically determined) or manual determination of genotyping data (number of repeats for each loci) was entered into Microsoft Excel (Redmond, WA, USA) tables and imported for further analysis into BioNumerics software (Applied Maths, St Martens, Belgium). Genetic distance analysis and cluster comparison were done by using a categorical variable index and the unweighted pair group method with arithmetic mean algorithm. If ≥2 isolates possessed identical VNTR signatures, they were considered to be clustered. For discrimination analysis, Hunter-Gaston diversity index (HGDI) was calculated as described (*31*) and used for comparison of the discriminatory power of VNTR typing for individual loci and for all loci taken together.

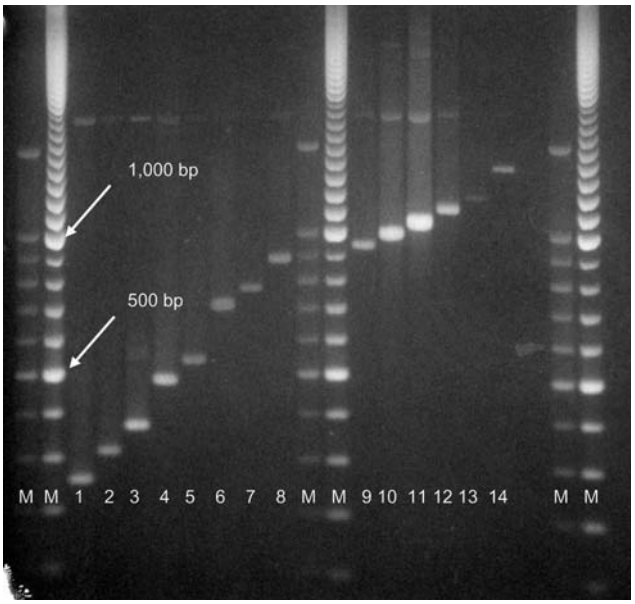

Figure. PCR analysis of VNTR3232 locus. Lane 1, 2 repeats; lane 2, 3 repeats; lane 3, 4 repeats; lane 4, 6 repeats; lane 5, 7 repeats; lane 6, 10 repeats; lane 7, 11 repeats; lane 8, 12 repeats; lane 9, 13 repeats; lane 10, 14 repeats; lane 11, 15 repeats; lane 12, 16 repeats; lane 13, 17 repeats; lane 14, 20 repeats. Lanes 1 - 5, strains other than Beijing; lanes 6- 14, Beijing strains. M, molecular weight markers.

Results

Baseline clinical and sociodemographic parameters of the 187 TB patients are shown in Table 1. MIRU-ETR analysis of the 187 isolates yielded 10 clusters of indistinguishable isolates and 58 unique patterns. The codes for the 15 loci were expressed in the following order: MIRU 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, 40, ETR-A, ETR-B, ETR-C. Cluster sizes varied from 2 to 75 isolates. The 2 largest clusters consisted of 31 strains with the 223325173533423 profile and 75 strains with the 223325153533423 MIRU-ETR profile, respectively, both consisting of Beijing strains (Table 2). For all strains, genotyping with the 15-MIRU–ETR loci set was more informative and had a higher discriminatory power than with spoligotyping, as expected (HGDI 0.747 for MIRU vs 0.572 for spoligotyping, data not shown).

The results of the allelic diversity analysis for all 187 isolates are summarized in Table 3. The discriminatory index for 5 loci (MIRU 26, MIRU 31, MIRU 39, MIRU 40, and ETR-A) exceeded 0.3; these loci were regarded as moderately discriminating according to definitions proposed in a recent study (*23*). Other loci were found to be less polymorphic, with HGDIs within the range 0 to 0.3; no polymorphism was registered for loci MIRU 24 and ETR-B. The maximal number of allelic variants (9) was registered for locus MIRU 10, although its discriminatory power was poor because of uneven distribution of isolates with different numbers of repeats.

Spoligotyping identified 129 isolates as belonging to the Beijing family (69.0%), a proportion similar to that previously reported for Samara Oblast (*7*,*18*). Most (123) of the Beijing isolates had the characteristic spoligotyping profile with 9 final spacers present in the DR region (35–43), whereas 6 isolates had incomplete profiles because they lacked spacer 40 (4 isolates) or 43 (2 isolates). Results of spoligotyping were verified by detection of the IS*6110* insertion in the *dnaA-dnaN* region. All 129 isolates yielded a PCR-product ≈2,000 bp, indicative of Beijing strains. Isolates belonging to strains other then

Table 1. Baseline clinical and sociodemographic parameters of 187 tuberculosis (TB) patients

Parameters	Isolates from civilian patients (N = 138), n (%)	Isolates from prisoners (N = 49), n (%)
Mean age, y (SD)	42.4 (14.3)	32.5 (10.6)
Sex		
Male	117 (84.8)	49 (100.0)
Female	21(15.2)	0
TB treatment		
New cases	105 (76.1)	36 (73.5)
Previously treated	33 (23.9)	13 (26.5)
HIV infected	4 (2.9)	5 (10.2)
Extensive lesions shown on radiograph	35 (25.4)	12 (24.5)
Contact with TB patient	54 (39.1)	25 (51.0)
Selected sociologic parameters		
Smoker	103 (74.6)	46 (93.9)
Alcohol consumption	121 (87.7)	39 (79.6)
Drug use	9 (6.5)	20 (40.8)

Beijing did not possess an insertion in this region and yielded an ≈550-bp PCR product.

The allelic diversity and HGDIs were calculated separately for Beijing strains (Table 4). These were subjected to VNTR analysis by using an additional panel of loci (0424, 0531, 1955, 1982, 2074, 2163a, 3232, 3239, 3336, and 3690; online Appendix Table). The allelic diversity analysis results calculated by using 25-MIRU loci and discriminatory indices are presented in Table 5.

Discussion

This study evaluated the discriminatory ability of VNTR analysis in a population of patients with TB, in which a predominant proportion of the infecting TB strains belonged to the Beijing family. This genetic group, along with the W family, have been recently shown to share similar characteristics, including absence of spacers 1–34 in the DR region of the *M. tuberculosis* genome (*14*,*15*,*17*). The latter characteristic, although well known and widely accepted, may not always be definitive for identification of Beijing/W strains alone, as spacers 35–43 are present in several other *M. tuberculosis* families. Moreover, some spacers (particularly 37, 38, and 40) are missing in certain Beijing/W isolates because of loss of the target for DRa and DRb primers caused by deletions or presence of IS*6110* insertions in the DR region (*32*,*33*).

Results of spoligotyping were confirmed by detection of an IS*6110* insertion between the *dnaA* and *dnaN* genes, which was found in all 129 Beijing isolates but not in the remaining isolates. Results of our analysis demonstrate 100% specificity of the method and its applicability for robust, rapid, and reliable identification of Beijing family strains.

In our study, 6 (4.7%) of 129 isolates identified as Beijing on the basis of spoligotyping had incomplete spoligotyping profiles with a missing spacer 40 or 43. As has been argued by Bifani et al. (*32*), W strains with missing spacer 40 are defined as W14 group, characterized by a specific IS*6110* RFLP pattern, high levels of drug resistance, and an additional repeat in ETR-D (MIRU 4) locus, i.e., MIRU-ETR profile 2**3**332515 (or 7) 3533423. However, we observed no differences in the number of repeats in the MIRU 4 locus for these 4 isolates. All 4 isolates were multidrug resistant strains. Of the 4 patients, all were male, 1 was HIV-infected, 2 were Russian (1 from Chechnya and 1 from Azerbaijan), and 2 had been vaccinated with BCG.

The data from the application of 15-MIRU–ETRs demonstrated the homogeneity and clonality of TB strains in this region of Russia: 135 (72.2%) of 187 strains were clustered into 10 groups, which indicates high rates of recent TB transmission in the Samara region. General trends for VNTR loci diversity and discriminatory power agreed (with some exceptions) with those reported previously (*20*,*22*,*23*,*34*). The overall allelic polymorphism and discriminatory power of the VNTR loci in this population were lower than that reported in previously published

Table 2. Prevalence of Beijing strains in clusters (15-loci MIRU-ETR analysis)*†

		No. repeats in MIRU-ETR loci														
		MIRU												ETR		
Cluster no.	Size	2	4	10	16	20	23	24	26	27	31	39	40	A	B	C
1	3	2	2	3	1	2	5	1	4	3	3	2	4	3	2	3
2	2	2	2	3	3	2	6	1	5	3	3	2	5	3	2	3
3	3	2	2	3	3	2	5	1	5	3	4	3	3	4	2	3
4	75	2	2	3	3	2	5	1	5	3	5	3	3	4	2	3
5	3	2	3	3	3	2	5	1	5	3	5	3	3	4	2	3
6	2	2	2	1	3	2	5	1	7	3	5	3	3	4	2	3
7	31	2	2	3	3	2	5	1	7	3	5	3	3	4	2	3
8	2	2	2	3	3	2	5	1	3	3	6	3	3	4	2	3
9	2	2	2	7	2	2	5	1	1	3	2	2	3	4	2	4
10	3	1	2	4	3	2	5	1	5	3	2	2	5	2	2	1

*MIRU, Mycobacterial interspersed repetitive units; ETR, exact tandem repeats.
†Clusters 3–8 had 100% Beijing strains; the other clusters had none.

Table 3. Frequency of occurrence of MIRU-ETR alleles and allelic diversity at each locus for all strains*

Locus	No. repeats										HGDI	No. AV
	0	1	2	3	4	5	6	7	8	9		
MIRU 2	0	11	172	3	0	0	1	0	0	0	0.151	4
MIRU 4	0	2	182	3	0	0	0	0	0	0	0.053	3
MIRU 10	0	2	3	166	15	3	1	5	1	1	0.206	9
MIRU 16	0	24	7	156	0	0	0	0	0	0	0.288	3
MIRU 20	0	1	186	1	0	0	0	0	0	0	0.011	3
MIRU 23	0	2	0	0	0	173	9	3	0	0	0.142	4
MIRU 24	0	187	0	0	0	0	0	0	0	0	0	1
MIRU 26	0	7	0	2	11	122	5	40	0	0	**0.526**†	6
MIRU 27	1	0	2	184	0	0	0	0	0	0	0.032	3
MIRU 31	0	1	20	31	7	124	4	0	0	0	**0.522**	6
MIRU 39	0	0	52	135	0	0	0	0	0	0	**0.404**	2
MIRU 40	2	3	7	147	14	13	1	0	0	0	**0.372**	7
ETR-A	0	3	18	28	138	0	0	0	0	0	**0.426**	4
ETR-B	0	0	187	0	0	0	0	0	0	0	0	1
ETR-C	0	11	9	157	10	0	0	0	0	0	0.288	4

*N = 187; MIRU, mycobacterial interspersed repetitive units; ETR, exact tandem repeats; HGDI, Hunter-Gaston diversity index; AV, allelic variants.
†**Boldface** loci showed at least moderate discriminative power as defined by Sola et al. (*23*) and were the most promising loci. Other loci provided poor discrimination or were monomorphic.

studies because of the large number of Beijing isolates in our test panel. This number reflects the actual prevalence of Beijing strains in Samara Oblast and in the few other regions in Russia for which the proportion of Beijing strains has been described (Tables 2–4).

The 15-MIRU–ETR profiles 223325153533423 or 223325173533423 were shared by 106 (82.2%) of the total number of analyzed Beijing strains with an HGDI of 0.625 for all 15 loci. Four loci (MIRU20, MIRU24, MIRU27, and ETR-B) were monomorphic for Beijing strains, and the only locus with sufficient discriminatory power for differentiating among Beijing family strains was MIRU 26. This finding is in marked contrast to the application of MIRU-ETR in other patient populations in which the discriminatory power of MIRU, used in conjunction with spoligotyping, was arguably almost comparable to RFLP IS*6110* (*20–23*,*35*). In these studies, HGDI values were subdivided into 3 groups, according to their ability to discriminate: poor (2, 20, 27), moderate (4, 16, 24, 39, ETR-B, ETR-C), and high (10, 23, 26, 31, 40, ETR-A) (*23*).

We expanded the panel of loci to improve discrimination among the Beijing isolates by using loci that had previously been reported to be highly polymorphic (*24–27*). The allelic diversity analysis and discriminatory indices are presented in Table 5.

The expanded set of VNTRs provided better discrimination than the original set of 15-MIRU–ETRs: 53 different profiles were identified, including 9 shared types in clusters (2–46 isolates each) and 44 unique patterns. The

Table 4. Frequency of MIRU-ETR alleles and allelic diversity at each locus for Beijing strains only*

Locus	No. repeats										HGDI	No. AV
	0	1	2	3	4	5	6	7	8	9		
MIRU 2	0	0	127	1	0	0	1	0	0	0	0.031	3
MIRU 4	0	0	126	3	0	0	0	0	0	0	0.046	2
MIRU 10	0	2	1	126	0	0	0	0	0	0	0.046	3
MIRU 16	0	1	0	128	0	0	0	0	0	0	0.016	2
MIRU 20	0	0	129	0	0	0	0	0	0	0	0	1
MIRU 23	0	0	0	0	0	128	0	1	0	0	0.016	2
MIRU 24	0	129	0	0	0	0	0	0	0	0	0	1
MIRU 26	0	0	0	2	1	89	0	37	0	0	**0.445**†	4
MIRU 27	0	0	0	129	0	0	0	0	0	0	0	1
MIRU 31	0	1	0	1	6	117	4	0	0	0	**0.176**	5
MIRU 39	0	0	1	128	0	0	0	0	0	0	0.016	2
MIRU 40	0	0	1	127	0	1	0	0	0	0	0.031	3
ETR-A	0	0	0	3	126	0	0	0	0	0	0.046	2
ETR-B	0	0	129	0	0	0	0	0	0	0	0	1
ETR-C	0	1	0	128	0	0	0	0	0	0	0.016	2

*N = 129; MIRU, mycobacterial interspersed repetitive units; ETR, exact tandem repeats; HGDI, Hunter-Gaston diversity index; AV, allelic variants.
†**Boldface** loci showed at least moderate discriminative power as defined by Sola et al. (*23*) and were the most promising loci. Other loci provided poor discrimination or were monomorphic.

Table 5. Frequency of an expanded set of 25 VNTR-MIRU alleles and allelic diversity for each locus for Beijing strains*†

Locus	No. repeats																		HGDI	No. AV
	1	2	3	4	5	6	7	8	9	10	12	13	14	15	16	17	20	25	26	
424	0	1	3	125	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.061	3
531	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	128	1	0.016	2
1955	1	4	1	122	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.105	4
1982	0	1	0	1	3	34	2	86	2	0	0	0	0	0	0	0	0	0	0	0.489	7
2074	0	129	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
2163a	0	1	0	0	2	5	0	1	117	1	2	0	0	0	0	0	0	0	0	0.177	7
3232	0	0	1	0	0	0	0	0	1	3	62	1	50	5	3	1	2	0	0	0.621	10
3239	0	2	126	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.046	3
3336	0	1	0	0	1	0	124	2	1	0	0	0	0	0	0	0	0	0	0	0.076	5
3690	1	128	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.016	2

*N = 129; VNTR, variable-number tandem repeats; MIRU, mycobacterial interspersed repetitive units; HGDI, Hunter-Gaston discriminatory index; AV, allelic variants.
†No loci had allelic variants with 11, 18, 19, and 21–24 repeats.

HGDI for 25 VNTR loci together was 0.870 (compared with 0.625 for the original set of MIRU-ETRs).

The most discriminatory individual VNTR were loci 3232 and 1982, with a large number of allelic variants (10 and 7, respectively) and HGDIs of 0.621 and 0.489, respectively. Other loci demonstrated poor discriminatory power (HGDI 0–0.2), and locus 2074 was monomorphic.

In our analysis of Russian Beijing isolates, 3 loci (MIRU 26, VNTR 1982, and VNTR 3232; 0.3–0.6) were sufficiently polymorphic for differentiation within the Beijing family. A relatively high degree of polymorphism in MIRU 26 has been previously reported (*20*,*23*,*36*). Loci 3232 and 1982 have been much less studied. Locus 3232 was shown to be more polymorphic in *M. tuberculosis* than in *M. bovis* (8 vs. 5 allelic variants, respectively) with moderate to high HGDI values (0.60). Locus 3232 displayed the highest discrimination power among 8 novel VNTR loci introduced by Roring et al. (*24*,*25*). The basis of polymorphism in loci MIRU 26, VNTR 3232, and VNTR 1982 is not completely clear. The first 2 loci are located in the intergenic regions of genes involved in metabolism of cell membrane components and transmembrane transport (locus VNTR 3232) and between genes *Rv2679* (possible *echA15*, enoyl-CoA hydratase) and *Rv 2680* (unknown protein) (locus MIRU 26). Locus VNTR 1982 is part of the *Rv1753c* (PPE24), which belongs to the group of genes encoding PPE proteins and which has been argued to be responsible for antigenic variation of *M. tuberculosis* (*37*). In our study, the degree of polymorphism at this locus was considerably higher than that previously reported for a panel of *M. bovis* isolates (*27*). By contrast, loci 2163a, 1955, 3336, and 3690, which were reported to be polymorphic for *M. bovis*, demonstrated low polymorphism in our study of *M. tuberculosis* Beijing isolates.

We speculate that the presence of variation at a small number of loci in genes likely to be involved in the earliest interactions of pathogen and host against a background of homogeneity at other loci suggests that these regions may be involved in the successful transmission of Beijing family strains. For the whole population studied, a small group of loci (VNTR 1982, VNTR 3232, MIRU 10, MIRU 26, MIRU 31, MIRU 39, MIRU 40, and ETR-A) offered the most discriminating panel and may be considered an essential part of the prospective universal panel of VNTR loci suitable for differentiation of *M. tuberculosis* isolates in different geographic settings with variable prevalence of highly conserved genotypes. The application of a limited number of loci (MIRU 10, MIRU 26, MIRU 31, VNTR 3232, and VNTR 1982) independently or in combination with other loci, may be a useful and rapid tool for differentiating strains within the Beijing family and for practical prospective genotyping and tracing of TB outbreaks in populations where the Beijing genotype predominates. Given the limited number of loci, analyzing this panel with a manual or automated approach is practical.

In conclusion, our study shows that by expanding the VNTR panel beyond the 15-MIRU–ETR loci described previously, discrimination can be substantially increased. This method may be used for typing *M. tuberculosis* isolates, even for populations in which a particular genetic group is dominant.

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State Plans for Containment of Pandemic Influenza

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This review assesses differences and similarities of the states in planning for pandemic influenza. We reviewed the recently posted plans of 49 states for vaccination, early epidemic surveillance and detection, and intraepidemic plans for containment of pandemic influenza. All states generally follow vaccination priorities set by the Advisory Committee on Immunization Practices. They all also depend on National Sentinel Physician Surveillance and other passive surveillance systems to alert them to incipient epidemic influenza, but these systems may not detect local epidemics until they are well established. Because of a lack of epidemiologic data, few states explicitly discuss implementing nonpharmaceutical community interventions: voluntary self-isolation (17 states [35%]), school or other institutional closing (18 [37%]), institutional or household quarantine (15 [31%]), or contact vaccination or chemoprophylaxis (12 [25%]). This review indicates the need for central planning for pandemic influenza and for epidemiologic studies regarding containment strategies in the community.

Much recent attention, public, governmental, and academic, has been focused on the possibility of an influenza pandemic, possibly arising from a mutated or genetically reassorted strain of the currently circulating avian influenza virus (H5N1). In the United States, state and local health departments are primarily responsible for detecting an outbreak and implementing the public health response. Accordingly, individual states and the US Department of Health and Human Services (HHS) have each recently released pandemic influenza plans and guidelines. These plans are now available (online Appendix Table 1, available from http://www.cdc.gov/ncidod/EID/vol12no09/06-0369_appT1.htm). Because state health departments are autonomous of federal control, their approaches to surveillance and containment are likely to vary. Thus, this review was undertaken to determine the extent of differences between states in their approaches to detecting and controlling pandemic influenza.

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State Procedures and Plans

Forty-nine states have Internet websites that include statewide pandemic influenza procedures and plans or, in a few instances, have simply addressed broad questions about state-based responses to pandemic influenza (Appendix Table 1). Often these plans, which are funded by a Centers for Disease Control and Prevention (CDC) preparedness cooperative agreement, were posted in the last half of 2005 or early 2006, and these documents are still largely in transition. US HHS has recently issued guidelines (*1*); however, specific planning, problem solving, and funding are still left to the individual states.

Almost all of the states' plans address a wide range of issues regarding command and control, surveillance, vaccination, antiviral drugs, communication, and emergency management and containment measures. The purpose of this review was to focus on community public health strategies, especially vaccination, surveillance and detection, and containment, which the various states develop as they refine their plans.

General Processes

Vaccination

In general, all 49 states with posted plans are in accordance with one another on vaccination priority strategies (online Appendix Table 2, available from http://www.cdc.gov/ncidod/EID/vol12no09/06-0369_appT2.htm). Many state plans indicated that they were constrained by the uncertainties of future vaccine and antiviral drug supply and effectiveness and the properties of a future influenza epidemic.

Nonetheless, general agreement exists, explicit or implicit, to provide vaccination during a pandemic that is

prioritized by those most likely to acquire, become ill, or die from pandemic influenza (Appendix Table 2). Generally, these guidelines were based on Advisory Committee on Immunization Practices (ACIP) recommendations (*2*) and HHS guidelines (*1*) if the latter were available at the time the state plan was drafted. The clear priorities involved the following groups: 1) healthcare workers (some states also include emergency responders such as emergency medical technicians, fire fighters, and police officers; political leaders; utility workers; transporters of vaccine; and vaccine manufacturers); 2) persons with respiratory, immunodeficiency, cardiovascular or other high-risk conditions; 3) persons ≥65 years of age (17 [35%] plans also consider vaccinating children 6–23 months of age); 4) household members, caregivers, and other close contacts of those ill or likely to be ill; and 5) all others.

The first 3 of the these groups were variously prioritized by the states as high-priority vaccine recipients, but all states consider these 3 groups as the highest priority. A few states, such as Maine, have estimated that 15%–20% of their populations would be in such high-priority groups to be vaccinated.

Surveillance and Detection

Of the 49 states that have posted their plans, all rely on the National Sentinel Physician Surveillance (NSPS), the nationwide 122 Cities Mortality Reporting System of pneumonia- and influenza-related deaths (*3*,*4*), or both. Forty-seven (96%) states also explicitly indicated they would evaluate reports of clusters or unusual influenzalike cases by local physicians, clinics, and institutions in their state, and 40 states (82%) indicated they would evaluate reports of laboratory-confirmed influenza from state health department and other laboratories (Appendix Table 2).

Although many states describe plans for enhanced surveillance during pandemic influenza, relatively few (12 [25%]) currently have or envision real-time syndromic surveillance of influenzalike illness (ILI) in persons seeking care at clinics or hospital emergency departments to detect the onset of pandemic influenza (Appendix Table 2). To our knowledge, the cities currently using syndromic surveillance are New York City, Washington, DC, Pittsburgh, and some cities in North Carolina and Virginia. In addition, the CDC BioSense program (*5*) plans to expand syndromic surveillance to 300 clinical sites by the end of 2006.

Eight states (16%), including California (especially in Los Angeles), New York, and Hawaii, are developing ways of screening incoming international travelers. However, current plans for other international entry points, such as Seattle, Portland, Chicago, and Atlanta, do not indicate any similar (foreign) international traveler quarantine and testing activities.

Containment Measures

The various state plans are markedly heterogeneous in their personal contact-avoidance measures and prophylaxis (Appendix Table 2). Most states outline pandemic influenza responses that do not include general and early encouragement of many specific personal avoidance steps, such as staying home from work and keeping sick children at home.

Seventeen (35%) states explicitly plan or are considering recommending (voluntary) personal social isolation on the community level, such as staying at home or keeping children at home if they feel sick. Eighteen (37%) other states cite federal or state regulations that indicate that health authorities may close schools, businesses, and other institutions during a severe outbreak, although they are not required to resort to such closures. However, 15 (31%) other states also indicate legal ability to quarantine persons, households, or institutions. (However, in some states such as Michigan, the efficacy of quarantine is directly questioned in their current document.)

Given the high cost and limited supply of neuraminidase inhibitor antiviral drugs and an uncertain supply and effectiveness of future vaccines, only 12 (25%) states plan or consider using either chemoprophylaxis (such as oseltamivir) or vaccination of household and other close nonhospital contacts in their plans to retard epidemic influenza.

Implications

The control of future pandemic or interpandemic influenza will necessarily rely on each individual state's plan to vaccinate persons and detect and contain this disease. Still, the current national (HHS) pandemic influenza plan presents only a categorization and listing of steps, rather than explicit direction for the states. This lack of central coordination can result in a patchwork of plans that will not adequately detect and control this or other respiratory disease pandemics.

Given the lack of clear guidance, coupled with the fact that no one knows when an influenza pandemic may strike, what its characteristics will be, and the effectiveness and quantity of strain-specific vaccine, the evolving state plans are nonetheless in agreement in adhering generally to ACIP and HHS guidelines for prioritizing vaccination (*1*,*2*). In general, the elderly, those with chronic diseases, and healthcare and infrastructure personnel will be prioritized to receive vaccination, and in approximately one third of states, young children will be prioritized to be vaccinated (*6*). We believe the estimate that such persons make up ≈15%–20% of the population in any state is reasonable (*1*). However, this vaccination strategy is predicated on preventing deaths from influenza, not stopping or retarding an epidemic or pandemic (*7*). Given that vaccine

adequate to cover the entire US population will not be available for several more years, the goal of reducing transmission would require much more vaccination than is available.

Regarding surveillance and detection, state plans are even more variable than they are about strategizing vaccinations. All states indicate that they plan to use the NSPS network and the nationwide 122 Cities Mortality Reporting System (*3*,*4*). In NSPS, each year ≈1,100 (55%) of ≈2,000 healthcare providers nationwide voluntarily report the number of weekly outpatient visits for ILI and submit specimens from a subset of patients to state public health laboratories for influenza virus testing. The 122 city-specific mortality reporting is unavoidably even more delayed. Neither system would likely detect a local outbreak of influenza <2 weeks into its establishment. Thus, some state and federal authorities are assessing the utility of syndromic surveillance of ILI (e.g., cough, headache, and fever) in emergency departments and clinics in test cities. Several such programs exist, and CDC's BioSense (*5*) includes plans to expand such early surveillance to 300 clinical sites. However, to our knowledge, no health authority feels confident that earlier detection of influenza by 1 to 3 weeks would necessarily lead to better control or substantial retardation of an outbreak.

Finally, confusion and lack of specificity exist in these posted state plans in proposing practical containment measures in the community. The national HHS Pandemic Influenza Plan (*1*) has several recommendations for infection control in the hospital setting but is weaker and nonspecific in other areas such as control of influenza in the community. For example, there is no agreed-upon definition of geographic clustering of cases or number of persons infected that will trigger the declaration of a pandemic. Much of this national plan suggests social distancing and respiratory (cough) etiquette and devotes much of its discussion to mask use. Accordingly, states are comparably nonspecific about community control plans. Vaccination or chemoprophylaxis of contacts is infrequently addressed, mainly because the vaccine supply is limited, as are the most effective antiviral drugs, the neuraminidase inhibitors oseltamivir and zanamivir, for the next several years. Even when these vaccines and drugs become available, considerable obstacles remain to detecting influenza promptly and getting vaccination or drug therapy to the contacts of influenza patients.

Several practical nonpharmaceutical containment steps need to be considered. For example, only approximately one third of the state plans are explicitly considering recommending self-isolation of adults with influenzalike symptoms and keeping children with such symptoms home from school and daycare. Even in this increasingly computer-based economy, in which a considerable percentage of persons can work from home most of the time, this simple stratagem is not addressed in most state plans. Other simple recommendations for use in the community, such as avoiding mass gatherings; shopping on off hours; and household and workplace strategies such as frequent hand washing, avoiding handshaking, and keeping towels separate, are often neglected in state plans.

Why are there these state plans so disparate? We believe some of the problem results from weak central (federal) direction, as has been a criticism of national bioterrorism preparedness (*8*). Fortunately, state and federal plans are still in flux, many are still in draft form (Appendix Table 1), and getting a clearer delineation of a basic plan that all states can follow is still possible. The US Secretary of HHS has been meeting with states (http://www.pandemicflu.gov/plan/convening.html) to review pandemic preparedness issues.

However, we also believe that answers are lacking to several key epidemiologic questions necessary for rational planning. What is the typical intrahousehold or institutional attack rate, and would vaccination or chemoprophylaxis of contacts retard or stem outbreaks? How well do masks work for pandemic influenza in the community, and when and for how long should they be used? Does closing a school or other institution actually reduce community-level illness and death? Does earlier detection of influenza in a community lead to behavior changes that could stem an outbreak? We know of no studies designed to address these and several other issues; e.g., the Models of Infectious Disease Agents Study (MIDAS) (*9*) has been forced to rely on estimates of household attack rates estimated >30 years ago (*10*,*11*) for a nonpandemic (H3N2) strain of influenza virus. Several state plans, such as California's, expressed frustration about this lack of information.

Thus, we believe that a revision of the national pandemic influenza plan, which despite unavoidable gaps in our knowledge, relies on professional and public health opinion to provide more uniform, specific, and practical influenza protection, avoidance and containment practices for pandemic and interpandemic influenza in the community would be helpful. We also believe it would be prudent to begin studies and, in the interim, create expert panels to determine if masks, school closings, social isolation, and several other nonpharmaceutical strategies would be useful in reducing the illness and death caused by pandemic influenza and its spread in the community.

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Human Bocavirus Infection among Children, Jordan

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Human bocavirus was detected in 57 (18.3%) of 312 children with acute respiratory infection (ARI) who required hospitalization in Jordan. It was also detected in 30 (21.7%) of 138 children with severe ARI, in 27 (15.5%) of 174 with mild or moderate disease, and in 41 (72%) of 57 with other pathogens.

Acute respiratory infection (ARI) is a major cause of illness and death worldwide (*1*). Although ARI is the third most common cause of death overall, in children it is the major cause of death outside the neonatal period; an estimated 2 million deaths occur in children <5 years of age, predominantly in developing countries (*2*). Viruses are a cause of upper and lower respiratory tract infections in children and several of them have been described. Among these, respiratory syncytial virus (RSV) is most important, both in terms of prevalence and effect (*3*). However, in recent years, several new viruses have emerged. These include human metapneumovirus (*4*), severe acute respiratory syndrome coronavirus (*5*) and human coronaviruses HKU1 and NL63 (*6*,*7*).

In 2005, Allander et al. reported detection of a new human parvovirus that they named human bocavirus (HBoV) (*8*). They detected this virus by constructing libraries of amplified DNA and RNA from supernatants of nasopharyngeal aspirates of children with ARI and removing nonviral nucleic acids by ultracentrifugation, microfiltration, and treatment with DNase. From this analysis, a novel parvovirus sequence was obtained. The complete genome sequence was determined and HBoV was characterized. The only other related bocaviruses are bovine parvovirus and canine parvovirus 1 (CPV-1). A PCR detection method was devised that targeted the noncapsid protein-1 (NP-1) gene, and virus was detected in 24 (3%) of 806 children with ARI in Sweden. We used the same PCR detection method to determine whether HBoV is a potential cause of ARI in children in Jordan.

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The Study

From December 2003 to May 2004, all children <5 years of age admitted to the pediatric wards of King Hussein Medical Centre (KHMC) and Queen Alia Hospital (QAH) in Amman, Jordan, were enrolled into the study after informed consent was obtained from parents or guardians. The study, which determined the etiology, inflammatory responses, and clinical effects of ARI was approved by the research ethical approval committee of the Royal Medical Services, Amman, Jordan. KHMC and QAH provide all hospital pediatric care for Amman and its surroundings.

Diagnosis of ARI and assessment of its severity was made by using World Health Organization (WHO) standard protocol for ARI based on the presence of cough, tachypnea, chest indrawing, and wheezing for <7 days (*9*). Severe disease was defined in children with a respiratory rate >60/minute and chest indrawing. Oxygen saturation (pO_2) was measured by using pulse oximetry (Nellor, Puritan Bennet, UK), and a $pO_2 \leq 85\%$ was used as the cutoff for giving supplementary oxygen. Nasopharyngeal aspirates (NPAs) were collected by instilling 1 mL sterile phosphate-buffered saline through a nasopharyngeal mucous extractor. The aspirate was frozen at –80°C and transported frozen to Liverpool for analysis.

DNA and RNA were extracted from aspirates by using commercial kits (Qiagen, Basingstoke, UK). PCR or reverse transcription PCR (RT-PCR) detection of influenza A and B viruses, parainfluenza virus 1–4 (*10*), human metapneumovirus, RSV (*11*), adenovirus, *Chlamydia* spp., and *Mycoplasma pneumoniae* (*12*) was performed according to previously published protocols. HBoV primers 188F (5′-GAGCTCTGTAAGTACTATTAC-3′) and 542 R (5′-CTCTGTGTTGACTGAATACAG-3′) that target the NP-1 protein gene and produce a 354-bp amplicon were used as described and modified by Allander et al. (*8*). Other potential respiratory pathogens such as rhinoviruses and coronaviruses were not investigated because they are associated primarily with upper respiratory infections.

A total of 326 children were enrolled in the study, but sufficient nucleic acid was extractable from 312 NPAs for detection of each potential respiratory pathogen. For the remainder, the volume of NPA was too small for extraction of both DNA and RNA Of these, 57 (18.3%) children were infected with HBoV (Table). The median age of HBoV-infected patients was 8 months and 29 (51%) were male, compared with a median age of 6 months and 156 (61%) male patients in the HBoV-negative patients ($p \geq 0.2$). HBoV was detected in 30 (21.7%) of 138 children with severe ARI and in 27 (15.5%) of 174 children with mild-to-moderate ARI ($p \geq 0.2$). However, only HBoV was detected in 13 (48%) of the 27 patients with mild-to-moderate ARI and with adenovirus (10 patients), RSV (2

Table. Acute respiratory infections associated with human bocavirus in Jordanian children*

Date	No. NPAs tested	No. positive (no. mixed infections)	% positive
Dec 2003	7	1 (1)	14
Jan 2004	95	18 (11)	19
Feb 2004	117	19 (15)	16.2
Mar 2004	62	8 (6)	12.9
Apr 2004	27	10 (7)	37
May 2004	4	1 (1)	25
Total	312	57 (41)	18.3

*NPAs, nasopharyngeal aspirates.

patients) *Chlamydia* spp. (1 patient), and RSV and adenovirus (1 patient) in the 14 remaining patients with mild-to-moderate disease. In patients with severe ARI in whom HBoV was detected, it was the only pathogen in 3 (10%) patients. In the remaining 27 cases, it was found as a mixed infection with RSV (9 patients), RSV and adenovirus (8 patients), RSV and *Chlamydia* spp. (2 patients), RSV and influenza A virus (1 patient), HMPV and *Chlamydia* spp. (1 patient) and adenovirus (6 patients). The median age was 3.5 months for those infected only with HBoV and 10 months ($p = 0.012$) for those co-infected with HBoV and other potential pathogens.

Direct sequencing (Lark Technologies, Essex, UK) was undertaken for 14 (25%) of the amplicons. Four amplicons had the same sequence as the original Swedish strain. Five variants were detected. One cluster (DNA Data Bank of Japan accession no. AB243566 available from www.ddbj.nig.ac.jp) contained 5 strains with mutations at codons 21 (R→K) and 59 (S→N). Another cluster (AB 243570) contained 2 strains with 1 mutation at codon 79 (S→N). Three other variants were detected with changes at codons 26 (R→K), 29 (Q→R), and 59 (S→N) (AB243568), codons 21 (R→K) and 79 (S→N) (AB243569), and codon 42 (R→Q) (AB243567), respectively. No connections were found between patients with different variants except for AB243570, in which 2 strains were isolated from 2 children at the same orphanage in Amman who came to the hospital on the same day. One had mild-to moderate-disease, and the other had severe disease.

Conclusions

We detected HBoV in 57 (18.3%) of 312 children with ARI severe enough to require hospital admission. HBoV was detected in 30 (21.7%) of those admitted who were classified according to WHO criteria as having severe ARI. Other reported prevalences are 24 (3%) of 806 pediatric samples in Sweden (*8*), 18 (5.6%) of 324 children <3 years of age in Australia (*6*), and 18 (5.7%) of 318 children <3 years of age in Japan (*13*). These data support an association between the virus and ARI.

As in the Australian study (*6*), mixed infections were common. In the Australian study, HBoV was detected with other potential respiratory pathogens in ≈56% of patients. In our study, the prevalence (72%) of mixed infection was even higher, occurring most often as a co-infection with RSV. HBoV was found as sole pathogen in 2% of cases of severe ARI and in 7.5% of mild-to-moderate ARI.

This study was conducted during the peak period of ARI in Jordan, and the prevalence of detection of HBoV ranged from 12.9% in March to 37% in April. However, larger cross-sectional studies and longitudinal studies of HBoV-infected children are needed to determine whether HBoV causes ARI, its effect on children, and its seasonality. In addition, HBoV, similar to some adenoviruses (*14*) and other human parvoviruses, may show persistent shedding after an initial acute infection.

Finally, we have also obtained evidence for variations in the HBoV NP-1 gene. In addition to the original Swedish strain, we found 5 variants with point mutations in the gene causing amino acid substitution in the deduced protein. What role this might play in HBoV pathogenesis and whether other genes encoding nonstructural protein 1 (NS-1) and virion proteins 1/2 (VP1/2) show similar variability are unclear. However, 2 CPV-1 strains showed 96.5%, 92.5%, and 97.5% homology in their NS-1-, NP-1-, and VP1/2-deduced proteins (*15*).

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Carriage of *Neisseria meningitidis* Serogroup W135 ST-2881

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Serogroup W135 ST-2881 meningococci caused a cluster of meningitis cases in Niger in 2003. Of 80 healthy persons in the patients' villages, 28 (35%) carried meningococci; 20 of 21 W135 carrier strains were ST-2881. Ten months later, 5 former carriers were still carriers of W135 ST-2881 strains. The serum bactericidal antibody activity changed according to carrier status.

Niger is located in the African "meningitis belt" (*1*). Until recently, meningococcal meningitis epidemics in Niger were caused primarily by *Neisseria meningitidis* serogroup A. Since the first epidemic in Africa, caused by *N. meningitidis* serogroup W135 (NmW135) in Burkina Faso in 2002, Niger has enhanced its microbiologic surveillance. Few laboratories perform etiologic diagnoses, but health staff can send frozen cerebrospinal fluid (CSF) specimens to the national reference laboratory, Centre de Recherche Médicale et Sanitaire (CERMES), for microbiologic determination by PCR (*2*,*3*).

In March and April 2003, the district of Illela reported 154 suspected cases of meningitis. The epidemic threshold of 10 cases/100,000 inhabitants/week was crossed at week 12. The incidence decreased by week 14, with no vaccination campaign (Figure).

Etiologic diagnosis was not made immediately, but 15 frozen and stored CSF specimens were retrieved in May. Among the 11 specimens with positive PCR results for *N. meningitidis*, 5 were NmW135 and 6 were NmA. All cases caused by NmW135 were reported by the Illela health center (14°27′N, 05°14′E) and were in patients living in 5 surrounding villages.

To understand the limited size of this cluster of NmW135 cases in a population never vaccinated against this serogroup, we surveyed the prevalence and duration of meningococcal carriage among inhabitants of patients' villages. We also assessed the seroprevalence and immunologic response induced by carriage.

The Study

Carriage studies carried out by CERMES were approved by the national ethics committee of Niger in February 2003. We conducted our first investigation in May 2003 in 4 of the 5 villages where the meningitis patients were living. In each village, we enrolled 20 consenting persons, 10 who lived in a patient's household, considered close contacts, and 10 who lived in a remote part of the village and had limited contact with patients (controls). The mean ages were 12.9 years (range 2–65 years) in the close contacts group and 12.1 years (range 6–25 years) in the other group.

Oropharyngeal swab specimens were immediately plated on chocolate agar. Plates were incubated at 37°C in a candle jar. From each culture that showed macroscopic evidence of *Neisseria*, 3 colonies were subcultured onto chocolate agar plates. Gram-negative oxidase-positive and catalase-positive cocci were then inoculated onto cystine trypticase agar. *N. meningitidis* serogrouping was performed by using specific antisera (Difco Laboratories, Detroit, MI, USA).

We collected a second oropharyngeal swab specimen from the same persons in February 2004. The swabs were processed as before. Meningococcal strains were sent to the WHO Collaborating Centre for Meningococci (Marseille, France) for serogroup confirmation, serotyping, multilocus sequence typing (MLST) (*4*), and pulsed-

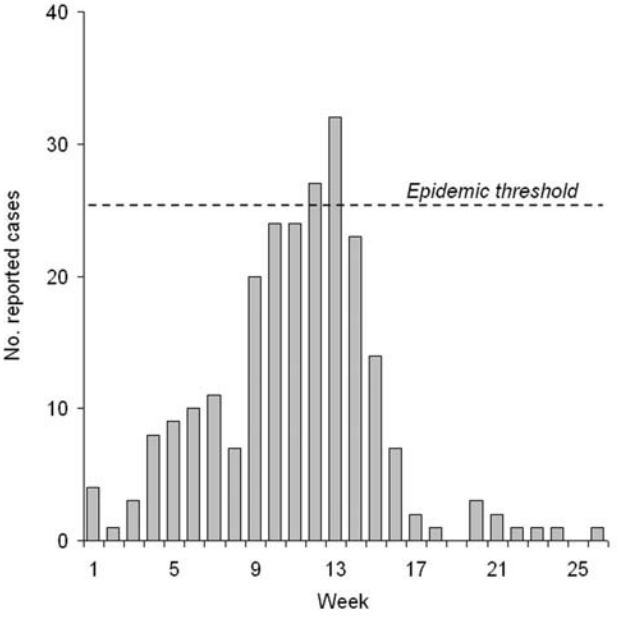

Figure. Weekly reports of suspected cases of meningitis, District of Illela, 2003.

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field gel electrophoresis (PFGE) (*5*). When 2 or 3 strains of the same serogroup were obtained from the 3 subcultured colonies, only 1 was sent for further analysis. An unpublished study by the meningococcus unit in Marseille showed that meningococci having the same PFGE fingerprint patterns belonged to the same sequence type (ST). However, not all meningococci belonging to the same ST had the same fingerprint pattern. Here, we attributed the same ST to all the isolates having the same fingerprint pattern, and all the isolates differing by ≥1 band were sequenced.

Serum samples were collected at the same time as the first and second throat specimens. We assessed the immunologic response to NmW135 by using the serum bactericidal antibody (SBA) assay, carried out according to the method of Maslanka et al. (*6*), and the standard operating procedure of the Vaccine Evaluation Department of the Manchester Medical Microbiology Partnership (VED/MMMP). We used the NmW135 ST-184 strain M01 240070 (W135:NT:P1.18–1,3) as a reference strain but also used a W135 (W135:NT:P1.5,2) ST-2881 local strain for comparison. Baby rabbit serum was used as a complement source. An SBA assay titer ≥8 for NmW135 was considered to reflect a protective immunity to this serogroup, according to the correlation established from NmC SBA (*7*). External quality control was conducted by VED/MMMP.

In May 2003, 28 (35%) of 80 villagers carried meningococci: 21 (26.3%) carried NmW135 strains, 2 (2.5%) carried NmY strains, and 5 (6.3%) had nongroupable strains. Carriage of NmW135 strains was not related to age or sex but was significantly more frequent among members of patients' households (40% vs 12.5%). Of the 28 recovered strains, 27 had an nontypable (NT):P1.5,2 phenotype irrespective of the serogroup. All 28 isolates were studied by using PFGE, and 10 were characterized by MLST. One of the 28 strains recovered in May 2003 was W135:2a:P1.2, ST-11; these characters were the same as those of the strain that was responsible for the epidemic in Burkina Faso in 2002. On the basis of MLST results for 9 strains that had the same PFGE pattern as all other isolates, 27 strains were attributed to ST-2881. Before 2003, ST-2881 strains had never been associated with invasive meningococcal disease (*8*,*9*).

We repeated the survey in February 2004. No new case of meningitis had occurred meanwhile. We could follow up 70 of the original participants. One (1.4%) carried NmY, 1 had a strain that could not be grouped, and 7 (10%) carried NmW135. Of these 7, five were already NmW135 carriers in May 2003, whereas another had carried a nongroupable strain. All 9 strains were ST-2881. Four of the 5 pairs of NmW135 strains had the same PFGE pattern in May 2003 and in February 2004, while the fifth pair differed in only 1 band. These 5 persons most likely carried these strains throughout the 10 months. Lastly, 2 additional persons (2.8%), who were not carriers in 2003, carried NmX, ST-181, in 2004.

MLST was successful in CSF specimens from 4 of the 5 patients. It showed that NmW135, which had caused the cases, also belonged to ST-2881.

The proportion of villagers with SBA assay titer ≥8 was not significantly different between close contacts and controls. The proportion of persons presumably protected against the local NmW135 ST-2881 strain increased from 25.8% to 41.9% ($p = 0.03$) within 10 months. Conversely, the proportion of persons with SBA assay titer ≥8 for the reference NmW135 ST-184 strain did not increase significantly (33.8% to 36.9%, $p = 0.8$). For persons with SBA assay titer <8 for the local strain in 2003, the proportion protected in February 2004 was significantly higher ($p = 0.04$) among those who were carriers in May 2003 (Table). For the reference strain, the difference was not significant between carriers and noncarriers (36.4% vs 15.6%, $p = 0.2$). Of the 6 NmW135 carriers in May 2003 who had an SBA assay titer <8 in 2003 and 2004 (Table), 2 were still NmW135 carriers in 2004.

Conclusions

Strains expressing the same polysaccharide (W135) gave slightly different SBA assay results. Antibodies against subcapsular antigens, likely PorA, may explain this finding. Studies have shown that carriage of >1 meningococcus genotype is rare. Therefore, the long-term carriage of NmW135 ST-2881 strains may have hampered colonization by another genotype, such as the hypervirulent NmW135 ST-11 strains. A recent study of religious pilgrims and their family contacts confirmed that NmW135 carriage could persist for several months (*10*). In May 2003, most carriers carried the same genotype, as observed during epidemics in which a single genotype usually emerges.

The carriage of isolates having the same subtype, ST, and fingerprint patterns but different polysaccharides, W135 and Y, suggests that capsule switching from W135 to Y is easy. This possibility is worrisome because the trivalent vaccine used in Africa to control NmW135 outbreaks does not contain the Y valence. NmW135 in meningitis patients in Niger was first reported in 1981 (*11*). The NmW135 clinical isolate of the ET-37/ST-11 clonal complex recovered in 2001 was the first to be typed (*12*). Since 2002, enhanced surveillance of meningitis showed the wide geographic spread of NmW135 in Niger (*13*). In 2003, ST-2881 represented >50% of NmW135 strains from patients in Niamey (*8*). This ST, which had never previously been associated with sporadic meningitis, has also been identified in Benin and Nigeria (*9*). The origin and

Table. Association between asymptomatic carriage status for W135 ST-2881 strains in May 2003 and protective immunity to the local strain in February 2004, in persons without protective immunity in May 2003*

Carrier of W135 strain, May 2003	Protective immunity to W135 ST-2881, February 2004		Total
	No (%)	Yes (%)	
No	26 (78.8)	7 (21.2)	33
Yes	6 (46.2)	7 (53.8)	13
Total	32	14	46

*According to serum bactericidal antibody assay; Fisher exact test, p = 0.04; ST, sequence type.

date of emergence of this ST are unknown because of lack of microbiologic surveillance outside Niamey before 2002. We report a cluster of cases that did not spread, despite the absence of a vaccination campaign and a high prevalence of long-lasting carriage. Until now, most strains with a genotype closely related to ST-2881 were carrier strains (*8*). ST-2881, which has a possibly lower virulence than the ST-11 strains, should be investigated in mice (*14*). Extensive circulation and asymptomatic carriage of ST-2881 strains in Niger may have prevented an epidemic by the virulent clonal complex ST-11. Carriage was significantly associated with development of a presumably protective immunity to the local ST-2881 strain. The association was not statistically significant for the reference ST-184 strain, but the limited sample size was not suitable for a high statistical power. Would the immunity induced by carrier NmW135 ST-2881 strains be sufficient to prevent an epidemic caused by the ST-11? Did the long-term carriage of a less virulent strain hamper colonization by a hypervirulent one? Addressing these 2 questions might contribute to understanding why the Burkina Faso outbreak did not hit Niger. This study highlights the importance of tracing NmW135 strains by MLST to monitor changes in the epidemiology of NmW135 in Africa.

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Lookback Exercise with Imported Crimean-Congo Hemorrhagic Fever, Senegal and France

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A patient with suspected malaria was hospitalized successively in 2 hospitals, first in Dakar, Senegal, then in Rennes, France, where tests diagnosed Crimean-Congo hemorrhagic fever. An international incident management group was set up in France and Senegal, which traced 181 contacts and analyzed 50 samples from 3 countries. No secondary cases were identified clinically.

A case of imported Crimean-Congo hemorrhagic fever (CCHF) was diagnosed in France in November 2004. Clinical features are described elsewhere (*1*). The patient was a 60-year-old French woman who had been hospitalized on November 4, 2004, in Dakar, Senegal, for severe influenzalike illness that had lasted 3 days. As her condition worsened, she was repatriated to Rennes University Hospital, France, on November 14. Serum samples obtained November 15 were sent to the National Reference Center for Hemorrhagic Fevers (NRCHV, Institut Pasteur, Lyon, France). Tests for anti-CCHF–specific immunoglobulin (Ig) M antibody by enzyme-linked immunosorbent assay (ELISA) and reverse transcription–PCR were positive. Tests for anti-CCHF IgG were negative.

After the NRCHV telephoned the diagnosis on November 22 (18 days after the date of first admission and 8 days after admission at Rennes University Hospital), control measures were taken immediately by an ad hoc group of persons and institutions responsible for prevention and control of infectious diseases. Contract tracing at Rennes Hospital was coordinated by the hospital's infection control committee, which identified all contacts among healthcare workers and persons who had handled or analyzed the patient's samples. The regional health authorities and epidemiologists from the Cellule Interrégionale d'Epidémiologie (CIRE) identified and followed-up all contacts in the patient's family. The Institut de Veille Sanitaire (InVS) documented the sequence of medical management and opportunities for accidental blood exposure to blood (*2*), informed all the healthcare worker teams that managed the patient's care, and coordinated efforts with the Institut Pasteur of Dakar in Senegal. The NRCHV made and reported the diagnosis. In Senegal, the Institut Pasteur was in charge of the investigation and contact tracing, which were carried out by epidemiologists with the assistance of entomologists and virologists.

The maximum duration of viremia was estimated to be 12 days, and the disease incubation period was estimated at 10 days (*3*,*4*). The group followed up potentially exposed persons for 10 days after a possible contact with the patient from November 4 through November 16, or 10 days after handling blood or tissue samples taken through November 16. A contact person was defined as anyone who was in direct contact (for example, family visitor, healthcare worker) with the patient or with samples taken from the patient between these dates. Contact persons did not undergo serologic screening, but all were informed of the need to self-monitor and were each followed up daily by a clinician either directly or by phone.

No accidental blood exposure was documented during healthcare procedures or handling of patient samples by any of the healthcare teams. Table 1 presents a summary of the sequence of events and occasions for secondary transmission. In Senegal, the patient had been admitted to a double room where she remained throughout her stay at hospital 1, except on the evening of her transfer to hospital 2. She had undergone several blood tests, received infusions, and had several visits from friends and colleagues. She had been transferred once to a dental clinic for bleeding gums. In hospital 2, she had been placed in a single room in the intensive care unit (ICU). Isolation procedures had been observed, and she had received no visitors. A central intravenous line had been placed, and blood samples had been obtained on several occasions. No isolation precautions were observed in the ambulance that had transferred her to the ambulance plane to Rennes. On the plane the patient's condition had been managed by a German medical team who had transfused whole blood through an existing catheter. The team had worn gloves but no masks. On arrival, she was transferred by ambulance from the Rennes airport to Rennes University Hospital.

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Table 1. Synopsis of phases, risks, and management of case of Crimean-Congo hemorrhagic fever*

Institution/phase	Place/date	Contacts, persons at risk	Measures	No. contacts (N = 181)	Responsible
Incubation phase	Senegal/ Oct–Nov 2	Contacts in Saly, Senegal	SOS Médecins Saly informed.	2	IPD and DIT-InVS
Before hospitalization	Dakar, Senegal/ Nov 2–4	Radiologist and his family	Radiologist informed	5	IPD and DIT-InVS
Hospitalization 1	Dakar, Senegal/ Nov 4–10	HCWs and laboratory personnel	Head physicians informed and agreed to verify staff health status	20	IPD and DIT-InVS
		Patients who shared the room	Head physicians informed and agreed to verify patient health status.	4	IPD and DIT-InVS
			Other physicians in health clinics welcoming expatriates informed		IPD and DIT-InVS
		Dental appointment	Dentist informed	1	IPD and DIT-InVS
		Visitors (friends)	Physician and radiologist informed	10	IPD and DIT-InVS
Ambulance transport 1	Dakar, Senegal/ Nov10	Personnel who transported the patient	Head physicians informed and agreed to verify staff health status	2	IPD and DIT-InVS
Hospitalization 2	Dakar, Senegal/ Nov 10–13	HCWs who provided care	Head physicians informed and agreed to verify staff health status	25	IPD and DIT-InVS
Ambulance transport 2	Dakar, Senegal/ Nov 13	Personnel who transported the patient	Head physicians informed and agreed to verify staff health status	2	IPD and DIT-InVS
Medical transport plane	Plane Dakar-Paris/ Nov 13–14	German team who provided care	Head physician informed and agreed to verify staff health status; German health authorities informed.	2	DIT-InVS
Ambulance transport 3	Rennes, France/ Nov 14	Personnel who transported the patient	Identification of contacts. Daily clinical follow up of contacts until Nov 26	2	Ddass 35 and CIRE Quest
Hospitalization 3	Rennes, France/Nov 14–24	ICU personnel who provided care	Identification of contacts. Daily clinical follow up of contacts until Nov 26	34	C-Clin Ouest, Ddass 35 and CIRE Ouest
		ID personnel who provided care	Identification of contacts. Daily clinical follow up of contacts until Nov 26	10	C-Clin Ouest, Ddass 35 and CIRE Ouest
		Personnel who transported the patient between wards	Identification of contacts. Daily clinical follow up of contacts until Nov 26	3	C-Clin Ouest, Ddass 35 and CIRE Ouest
		Laboratory personnel	Identification of contacts. Daily clinical follow up of contacts until Nov 26; identification/tracing of samples	50	C-Clin Ouest, Ddass 35 and CIRE Ouest
		Patient's family who visited	Identification of contacts. Daily follow up of contacts by telephone until Nov 26	9	C-Clin Ouest, Ddass 35 and CIRE Ouest

*HCW, healthcare workers; ICU, intensive care unit; ID, infectious diseases department; Nov 26; IPD, Institut Pasteur, Dakar; DIT, Département International et Tropical; INVS, Institut de Veille Sanitaire; CIRE, Cellule Interrégionale d'Epidémiologie.

While in ICU in the Rennes Hospital, isolation precautions were observed (strict adherence to standard precautions [*5*]; masks; single room). Visitors, however, were allowed. The masks were simple surgical masks, and the room was not a negative-pressure room. Blood samples were obtained repeatedly.

As her clinical status improved, the patient was transferred to a single room in the infectious disease department 2 days later. Again, standard precautions were strictly observed. The first biologic samples were sent to the laboratory in observance with standard procedures but with no particular additional precautions.

The group identified 71 patient contacts in Senegal and 2 (air ambulance transfer personnel) in Germany (Table 1). At the Rennes Hospital, 44 staff members in ICU or infectious disease departments, 50 laboratory personnel, and 3 persons responsible for bedside radiography or patient transfers were identified and contacted (Table 2). A total of 50 samples were identified and later destroyed.

Within 36 hours, the international team had identified 181 contacts among healthcare workers, visitors, fellow patients, and ambulance drivers in France, Germany, or Senegal and immediately informed the head physicians of each team that had cared for the patient since the date of symptom onset. No accidental blood exposure was identified, but extensive casual contact was documented. No secondary cases were identified clinically in any of the 3 countries.

The first symptomatic CCHF case in Senegal was identified in 2003 (*6*), but seroprevalence studies indicated that CCHF virus has been circulating there since 1969 (*7*,*8*). Another unrelated patient with a confirmed case, who had stayed in the same coastal area as our index case-patient in late November 2004, also became ill and exhibited fever and severe bleeding. She died 4 days later, causing no secondary cases among ≥4 healthcare workers and 4 friends or colleagues who cared for her, including one who sustained extensive accidental blood exposure but received ribavirin (P. Nabeth, pers. comm.).

Table 2. Possible patient contacts among Rennes Hospital personnel, by occupation

Work unit	Nurses	Physicians/ medical students	Nurses' aides	Ambulance staff	Technicians	Total
Intensive care unit	7	10	17	0	0	34
Infectious diseases department	3	4	3	0	0	10
Laboratories	0	1	10	0	39	50
Transportation and radiology	0	0	0	2	1	3
Total	10	15	30	2	40	97

This investigation of a case of imported viral hemorrhagic fever in France raised several issues. First, the patient did not have Lassa fever, unlike the diagnosis in most imported cases of viral hemorrhagic fever in industrialized countries (*9*). Second, the illness did not occur in a major French metropolitan center with an international airport that was home to a large community of migrant workers such as Paris, Lyon, or Marseille. The third issue is that of transmission and prevention of secondary cases. Suspected diagnosis was not announced by the team in Senegal before transfer. Therefore only standard precautions were observed during transfer or during this patient's care or the management of her blood and tissue samples. The patient was transferred and hospitalized in France 10 days after the onset of the first clinical signs. The absence of secondary cases could therefore be due to low levels of viremia because by the time the patient arrived in France the infection was nearing its end. However, no cases were described in Senegal either, where the patient was also hospitalized and also underwent invasive procedures in less stringent isolation conditions during a phase of high levels of viremia. The observance of standard precautions was therefore sufficient to prevent clinical secondary cases in Senegal, Germany, and France, as extensively described in developing or industrialized country settings (*10*,*11*). Observance of standard precautions has greatly improved in France and elsewhere during the last decade, as shown by the documented reduction of occupational infections and accidental blood exposures in French hospitals (*12*,*13*). These factors may have played a decisive role in the absence of clinically patent secondary transmission.

Hemorrhagic fever viruses leave no room for complacency. The risk for transmission and available means of prevention, however, are becoming increasingly well documented. This first imported case of CCHF in an industrialized country should lead French health authorities to review available evidence and reexamine the justification and applicability of the existing, stringent recommendations for the management of many patients in whom viral hemorrhagic fever is one of several possible diagnoses.

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Japanese Encephalitis Outbreak, India, 2005

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An outbreak of viral encephalitis occurred in Gorakhpur, India, from July through November 2005. The etiologic agent was confirmed to be Japanese encephalitis virus by analyzing 326 acute-phase clinical specimens for virus-specific antibodies and viral RNA and by virus isolation. Phylogenetic analysis showed that these isolates belonged to genogroup 3.

An epidemic of viral encephalitis was reported from July through November 2005 in Gorakhpur, Uttar Pradesh, India. It was the longest and most severe epidemic in 3 decades; 5,737 persons were affected in 7 districts of eastern Uttar Pradesh, and 1,344 persons died (*1*). Japanese encephalitis virus (JEV) is the most common cause of childhood viral encephalitis in the world; it causes an estimated 50,000 cases and 10,000 deaths annually (*2*,*3*). JEV is endemic in the Gorakhpur and Basti divisions of eastern Uttar Pradesh. The geographic features of this region are conducive for the spread of JEV; an abundance of rice fields and a bowl-shaped landscape allow water to collect in pools. Heavy rains saturated the ground in 2005, which caused ideal breeding conditions for mosquitoes that transmit the virus from pigs to humans. In addition, high temperature and relative humidity provided a suitable environment for JEV transmission.

We report in-depth investigations of JEV-specific antibodies, virus isolation, and demonstration of viral RNA in 326 febrile patients with encephalitis symptoms who were admitted to B.R.D. Medical College, Gorakhpur. Further molecular epidemiologic studies were performed to establish the genetic relatedness of the viral strain associated with this epidemic.

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The Study

A total of 326 clinical samples (185 blood and 141 cerebrospinal fluid [CSF]) were collected from the 326 patients who had a diagnosis of encephalitis. Two sets of blood samples, with and without anticoagulant, were collected for virus isolation and serologic tests. All serum and CSF samples were screened for JEV-specific immunoglobulin M (IgM) and IgG by using an in-house dipstick ELISA that incorporated nitrocellulose as the solid phase. Purified viral antigen was obtained from culture supernatant of infected $C_{6/36}$ cultures by sucrose density gradient ultra centrifugation (*4*–*6*). Results were confirmed by using an in-house IgM capture ELISA (*7*).

JE-specific RNA was detected by using the Access quick one-step reverse transcription (RT)–PCR kit (Promega, Madison, WI, USA) with the primer pairs JED3S: ATG CGC GGA TCC GAC AAA CTG GCC CTG AA (1839–1867) and JED3C: GGG GAA GCT TCG TGC TTC CAG CTT TGT CC (2193–2165) on the basis of the sequence in domain III of the E gene of strain JaOArS982 (*8*).

Virus isolation was attempted in $C_{6/36}$ cells (*4*) from RT-PCR– and IgM-positive serum and CSF samples according to standard protocol (*5*). Double-stranded sequencing of domain III of the E gene of JEV was performed on an ABI 310 sequencer (Applied Biosystems, Foster City, CA, USA) with the BigDye Terminator cycle sequencing ready reaction kit. The phylogenetic tree was constructed with the neighbor-joining method with bootstrap analysis of 1,000 replicates with the MEGA version 2.1 program (*9*).

Rural populations between the ages of 3 months and 15 years were affected; almost 50% of children 6–10 years of age were affected, and 35% of children ≤5 years of age were affected. The epidemic affected boys and girls at a ratio of 1.9 to 1. The overall case-fatality ratio was 23%. Children dominated the case load because most adults in the area are immune to the virus. The trend of the epidemic showed that most cases were reported from the first to third weeks of October. Clinical history showed that all patients had fever (temperatures 38.5°C–40°C); prominent symptoms included severe headache, convulsions, and vomiting, leading to paralysis, coma, and death.

Analysis indicated an overall positivity of 50% of serum samples and 30% of CSF samples. The antibody profile of the serum samples showed 23% IgM, 19% IgG, and 7% both IgM and IgG positivity, compared with 26% IgM, 4% IgG, and 1% both IgM and IgG positivity in CSF samples. A total of 9% of CSF samples were positive for JEV-specific RNA (355-bp amplicon) as determined by RT-PCR. All these RT-PCR–positive CSF samples were also positive for IgM. None of the serum samples were positive by RT-PCR for viral RNA. Adding RT-PCR– and IgM-positive samples to $C_{6/36}$ cells yielded 7 JEV isolates

from IgM-positive CSF samples only, as confirmed by ELISA and RT-PCR. The antibody profile of the RT-PCR– and isolation-positive samples is depicted in Table 1.

Further analysis of a 355-nucleotide sequence in domain III of the E gene of these isolates showed >95% homology with JEV on BLAST search. On comparison with 24 other geographically diverse JEV isolates (Table 2), all JEV isolates sequenced in this study were closely related (≥99% homology). The isolates from this outbreak showed a nucleotide sequence identity of 95.6% and 94.6% with prototype JEV (Nakayama strain) and the first Indian JEV (isolated from Vellore in 1956), respectively. The dendrogram showed that the JEV isolates responsible for the 2005 Gorakhpur epidemic belong to genogroup 3 (G3) but form a cluster separate from earlier Indian isolates (Figure).

Table 1. Antibody profile of RT-PCR– and virus isolation–positive samples, 2005 Japanese encephalitis virus outbreak in India*

Serial number	IgM	IgG	RT-PCR	Virus isolation
1	+	–	+	–
2	+	–	+	–
3	+	–	+	–
4	+	–	+	–
5	+	–	+	–
6	+	–	+	–
7	+	–	+	–
8	+	–	+	–
9	+	–	+	–
10	+	–	+	–
11	+	–	+	–
12	+	–	+	–
13	+	–	+	–
14	+	–	–	+
15	+	–	–	+
16	+	–	–	+
17	+	–	–	+
18	+	–	–	+
19	+	–	–	+
20	+	–	–	+

*RT, reverse transcription; Ig, immunoglobulin.

Conclusions

The Gorakhpur district of Uttar Pradesh, which shares a border with Nepal and Bihar, has been experiencing periodic outbreaks of JEV since 1978. The virus cannot usually be isolated from clinical specimens, even with the best laboratory facilities, probably because of low levels of viremia and the rapid development of neutralizing antibodies. The diagnosis is therefore usually based on the presence of antibodies. The IgM capture ELISA for serum and CSF has become the accepted standard for diagnosing

Table 2. Japanese encephalitis viruses compared for sequence analysis*

Sl no.	Virus ID no.	Year of sample collection	Country	Source	GenBank accession no.
1	G8924	1956	India	Mosquito	U70394
2	826309	1982a	India	Human brain	U70403
3	NA	1982b	India	Human brain	U03689
4	733913	1973	India	Human brain	Z34095
5	GP78	1978a	India	Human brain	AF075723
6	782219	1978b	India	Human brain	U70402
7	7812474	1978c	India	Human brain	U70387
8	P20778	1958	India	Human brain	Z34096
9	NO	1995	Australia	Human serum	L43566
10	SA14	1954	China	Mosquito	U14163
11	JKT7003	1981a	Indonesia	Mosquito	U70408
12	JKT9092	1981b	Indonesia	Mosquito	U70409
13	JKT5441	1981c	Indonesia	Mosquito	U70406
14	Nakayama	1935	Japan	Human brain	U03694
15	JaOH0566	1966	Japan	Human brain	AY029207
16	JaNAr0590	1990	Japan	Mosquito	AY427795
17	95-167	1995	Japan	Pig blood	AY377579
18	JaNAr0102	2002	Japan	Mosquito	AY377577
19	K91P55	1991	Korea	Mosquito	U34928
20	WTP-70-22	1970	Malaysia	Mosquito	U70421
21	691004	1969	Sri Lanka	Human brain	Z34097
22	86VN207	1986	Vietnam	Human brain	AY376461
23	89VN49	1989	Vietnam	Human brain	AY376462
24	02VN22	2002	Vietnam	Pig blood	AY376465
25	GP14†	2005	India	Human CSF	NS
26	GP48†	2005	India	Human CSF	NS
27	GP55†	2005	India	Human CSF	NS
28	GP67†	2005	India	Human CSF	NS
29	GP82†	2005	India	Human CSF	NS

*NA, not available; CSF, cerebrospinal fluid; NS, not submitted.
†Sequenced in this study.

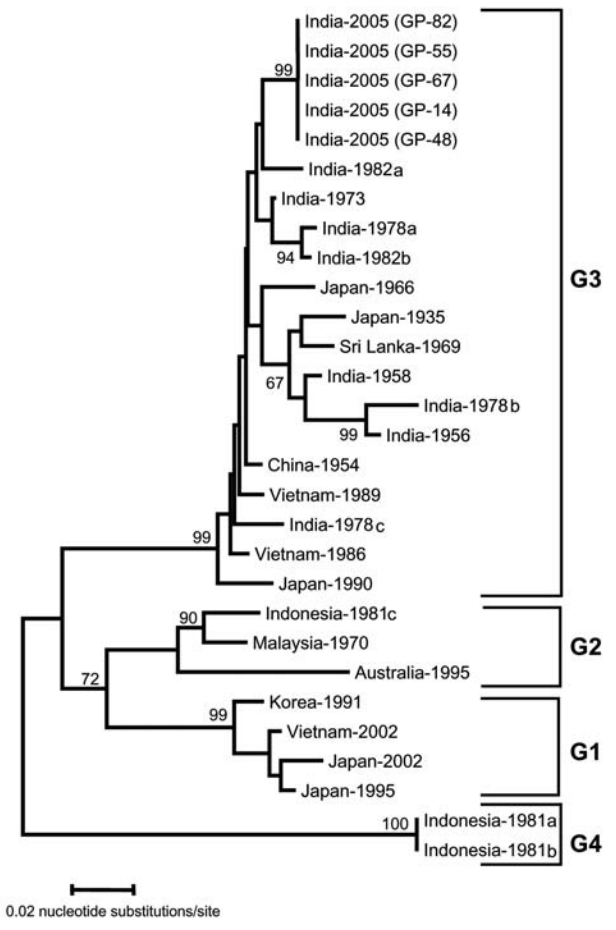

Figure. Sequence phylogeny based on partial E gene sequence of Japanese encephalitis virus isolates from the Gorakhpur epidemic, with reference to other Southeast Asian isolates. The tree was generated by neighbor-joining method. Each strain is abbreviated with the country of origin, followed by the year of isolation. Bootstrap values are indicated at the major branch points.

JEV.

The presence of only IgG antibodies in 19% of the patients indicated exposure to JEV infection in the past. This finding was expected because JEV is endemic to northern India, particularly Gorakhpur, and several large JEV epidemics have occurred in the past decade. In the present study, only 13 CSF samples (9%) were positive by RT-PCR. Seven virus isolates were obtained from IgM-positive CSF samples that did not yield RT-PCR amplicons before cultivation. Similar variations in virus detection and isolation have been reported (*10,11*); these findings underscore the sensitivity of cell culture systems for amplification of viable virus. Furthermore, the inability to detect genomic RNA or isolate virus from serum samples was striking and highlights the need for CSF sampling for both clinical diagnosis and epidemiologic studies.

We also investigated the molecular epidemiology of the outbreak by comparative sequence analysis of the isolates obtained in this study with reference strains of JEV. Domain III of the E gene was targeted for this purpose because this is the region under immune selective pressure, and it exhibits sufficiently rapid mutation to show evolutionary and epidemiologic relationships (*12–14*). We determined the partial sequence of these isolates directly from clinical samples without risk of altering the genome by passage in vitro. The dendrogram showed that the G3 of JEV is still circulating in India. However, compared with isolates from 1956 to 1988, recent isolates form a separate cluster. Frequent introduction of new virus genotypes through bird migration has led to shifts in circulating genotypes in neighboring Asian countries, including Japan, Vietnam, China, Korea, Sri Lanka, and Malaysia (*3,15*). Therefore, detailed and continuous epidemiologic surveillance is warranted to monitor the incursion and spread of JEV genotypes in India, which will allow effective control and management strategies to be undertaken at the earliest opportunity.

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Mycobacterium bovis Infection, Lyon, France

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In a 5-year retrospective study, we used spoligotyping and mycobacterial interspersed repetitive units to type 13 strains of *Mycobacterium bovis* isolated from human sources. Despite the relatively high incidence of human tuberculosis caused by *M. bovis* (2%), these tools showed no clonal evolution and no relationships between the isolates.

Mycobacterium bovis belongs to the *M. tuberculosis* complex (MTBC) and has a wide host range, infecting animals and occasionally humans. *M. bovis* has been a historical source of tuberculosis (TB) in humans infected through drinking contaminated unpasteurized milk or inhaling aerosols produced by diseased farm animals. Due to a national program of TB control, the incidence of *M. bovis* in France has dramatically decreased in cattle herds, falling from 10% in the1960s to 0.09% in 1998, and in humans, falling from 1.5% of TB cases in the 1960s to 0.5% (0.07/100,000) in 1995 (*1*,*2*). We describe 13 (2 were BCG strains) of 555 MTBC strains isolated from human samples (2% of incidence; we did not quantify the BCG strains), in Lyon, France, over a period of 5 years. Despite the small number of patients, our study shows a relatively high local incidence of infections caused by *M. bovis*. Advances in molecular typing have improved our understanding of the dissemination of *M. bovis* and helped improve our ability to distinguish among strains. Spoligotyping and mycobacterial interspersed repetitive units–variable-number tandem repeats (MIRU-VNTR) are now considered standard alternative molecular techniques (*3*,*4*). Both are PCR-based techniques that evaluate the polymorphism of the tandem repeat copy number at several loci and have been used to identify different strains of *M. bovis* (*5*,*6*). We used these molecular methods to identify different strains of *M. bovis*.

The Study

From 2000 to 2005, positive cultures were obtained from 13 patients with a diagnosis of *M. bovis* infection. The strains were screened by using *pncA* gene for resistance to pyrazinamide sequencing, and all displayed the 169 C→G mutation (*7*). To differentiate between *M. bovis* and *M. bovis* BCG, we tested for the presence or absence of the region of difference 1 because the absence of this region is a specific marker of BCG strains (*7*,*8*). Spoligotyping was performed in accordance with Kamerbeek guidelines, and the data were compared with the Institute Pasteur (IP) Spoligotype Database and with the International *M. bovis* Spoligotype Database (*9*,*10*). We performed MIRU-VNTR typing as described by Supply et al. (*11*,*12*).

Patient age, sex, sample site, and country of birth are provided in Table 1. Most of the clinical samples were from lymph nodes (n = 6). Others samples were from urine (n = 2), lung (n = 1), sputum (n = 1), cerebrospinal fluid (n = 1), ascitic fluid (n = 1), and synovial fluid (n = 1). Patient SO, who was 4 years old when his condition was diagnosed, had been born in France, but he spent months in Algeria with his grandmother who was ill with TB. Patient GD had a history of BCG-disseminated infection after being vaccinated with a BCG strain when he was 1 year of age. His condition had also been diagnosed as a familial form of septic granulomatosis, and he was immunocompromised. The strain was isolated only after he underwent lymph node resection at the age of 17. The bacillus isolated was an *M. bovis* BCG strain. Patient BL had undergone immunotherapy with a BCG strain for bladder cancer, and a BCG infection of the bladder developed.

The results of spoligotyping and MIRU are shown in Table 2. Spoligotype profiles were typical of *M. bovis* with the absence of spacers 3, 9, 16, and 39–43 (*1*,*13*). Four distinct patterns were identified; the main one corresponded to spoligotype 482 in the IP database (70% of strains); both BCG strains exhibited this pattern. Others patterns represented were spoligotype 481 (2 strains) and 2 that were not included in the IP database (although one was identified as SB0914 in the international spoligotype database). These 2 spoligotypes (481 and 482) have been reported by Haddad et al. as the ones most commonly seen in bovine TB in France (*1*). Patient MB's spoligotype was not found in the databases, likely because of its origin (this patient was born in Djibouti), and it could be native to Africa.

MIRU typing identified 12 individual patterns; 2 strains possessed the same MIRU patterns but not the same spoligotype. Both BCG strains showed the same pattern, except at locus 4 (*14*). Patient BL was found to have a BCG strain with 1 copy on locus 4. This profile is very similar to that of the Connaught strain used for the treatment of bladder cancer, which also has 1 copy at locus 4. Patient GD's strain of BCG had 2 copies at locus 4. This characteristic is similar to that of the BCG strain used for human vaccination in France (Mérieux strain derived from the Glaxo 1077 strain) (*14*).

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Table 1. Patient data, *Mycobacterium bovis* infection, Lyon, France, 2002–2005*

Name	Age, y	Sex	Sample source	Country of birth
SO	4	M	Cervical lymph node	France
GD	17	M	Cervical lymph node	France
BS	35	F	Lymph node	Algeria
MB	36	M	Cervical lymph node	Djibouti
KA	38	M	Mediastinal lymph node	Morocco
GA	53	F	Urine	Algeria
PC	53	M	CSF	France
TG	54	M	Synovial fluid	France
FJ	59	F	Cervical lymph node	France
OM	71	M	Lung biopsy	France
GA	73	M	Ascites fluid	France
BL	78	M	Urine	France
RM	89	M	Sputum	France

*CSF, cerebrospinal fluid.

Conclusions

This 5-year study of human *M. bovis* infections in humans leads to 3 main conclusions. First, we observed a relatively high incidence of this disease: 2% of TB cases were caused by *M. bovis*, compared with 0.5% reported 10 years earlier and ≈1% reported in England by Smith in 2004 (*15*). Second, in France TB caused by MTBC occurs mainly in patients born abroad (55%), whereas in this study 70% of TB due to *M. bovis* occurred in French-born patients (4 of the patients had been born abroad). Therefore, human disease due to *M . bovis*, in contrast with that due *to M. tuberculosis*, appears to be predominantly indigenous in France, according to our study. However, we must note that human *M. bovis* infection varies throughout the world, even in industrialized countries, as reported in MMWR in 2005 when patients infected in New York were young persons born in Mexico or children of Mexican-born parents (*16*). Finally, we should note that French patients with *M. bovis* infections, in contrast to patients born abroad, were usually ≥50 years of age and sought treatment for a torpid infection. Measures to reduce bovine TB and the human transmission of *M. bovis* began in the 1950s. The disease was due to the reactivation of a past infection that had been acquired before milk pasteurization rather than a primary infection. Few cases have been reported in French-born children, which is in accordance with the effectiveness of preventive measures and their long-term effect. We cannot tell whether this is an emerging or a reemerging disease, but *M. bovis* is clearly still responsible for human TB. Global monitoring is required to confirm the progress of the disease and perhaps to explain why it is (re)emerging. In summary, we found the combination of spoligotyping and MIRU-VNTR to be a useful tool for identifying *M. bovis* infections and for determining whether patients were infected with the same strain. In our population of patients in Lyon, France, we did not detect any clonal epidemiologic features for *M. bovis* disease.

Dr Mignard is a clinical scientist in the Department of Bacteriology, Lyon Sud University Hospital, Lyon, France. Her research interests include TB and molecular methods for identifying mycobacteria.

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Table 2. Spoligotyping and MIRU typing results

Patient	Strain	Spoligotype IP*	International spoligotype†	MIRU type‡	Spoligotypes
SO	*M. bovis*	481	SB0121	23232 42533 22	■■□■■■■■□■■■■■■□■■■■□■■■■■■■■■■■■■■■■■□□□□□
GD	*M. bovis* BCG	482	SB0120	22232 42533 22	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■□□□□□
BS	*M. bovis*	482	SB0120	23232 42512 22	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■□□□□□
MB	*M. bovis*	Not present	Not present	23232 42533 22	■■□□□□□■□■■■■■■□■■■■■■■□□□■□□□□□□□□□□■□□□□□
KA	*M. bovis*	482	SB0120	22232 52523 22	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■□□□□□
GA	*M. ovis*	481	SB0121	23232 42333 22	■■□■■■■■□■■■■■■□■■■■■□■■■■■■■■■■■■■■■■■□□□□□
PC	*M. bovis*	482	SB0120	23232 42423 22	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■□□□□□
TG	*M. bovis*	482	SB0120	23232 42523 22	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■□□□□□
FJ	*M. bovis*	482	SB0120	22232 42523 22	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■□□□□□
OM	*M. bovis*	Not present	SB0914	23242 42533 22	■■□■■■■■□□□■■■■□■■■■■■■■■■■■■■■■■■■■■■■■□□□□□
GA	*M. bovis*	482	SB0120	23202 42523 22	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■□□□□□
BL	*M. bovis* BCG	482	SB0120	21232 42533 22	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■□□□□□
RM	*M. bovis*	482	SB0120	22231 43533 22	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■□□□□□

*IP, Pasteur Institute Spoligotype Database; available from http://www.pasteur-guadeloupe.fr/tb/spoldb3/spoldb3.pdf
†International Spoligotype Database; available from http://www.Mbovis.org (*10*).
‡Mycobacterial interspersed repeat units (MIRU) type described by Supply et al. (*11*; available at www.ibl.fr/mirus/mirus.htm).

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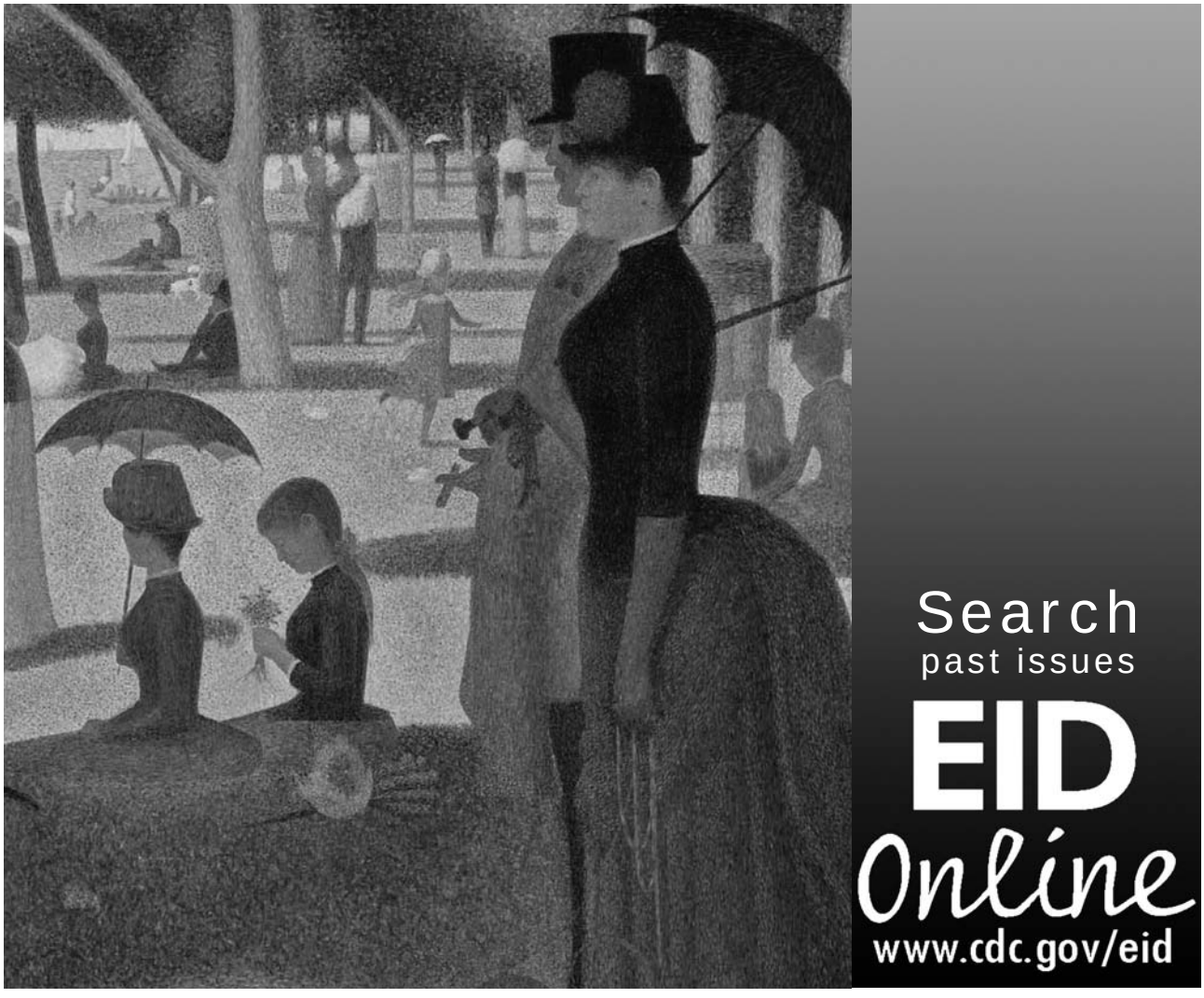

Extrapulmonary Tuberculosis among Somalis in Minnesota[1]

R. Bryan Rock,*† Wendy M. Sutherland,‡ Cristina Baker,*† and David N. Williams,*†

To analyze extrapulmonary tuberculosis in Somalis living in Minnesota, we reviewed surveillance and public health case management data on tuberculosis cases in ethnic Somalis in Minnesota from 1993 through 2003. The presence of these recent immigrants substantially affects the local epidemiology and clinical manifestation of tuberculosis.

Although the incidence of tuberculosis in the United States has declined each year since 1993, tuberculosis remains an important infectious disease in the United States and worldwide. In Minnesota, the incidence of tuberculosis increased during the 1990s and peaked at 4.9 cases per 100,000 population in 2001. From 2001 through 2005, 81% of tuberculosis cases in Minnesota occurred in foreign-born persons; this finding can largely be attributed to dynamic immigration patterns that have included an influx of persons from areas of the world where tuberculosis is endemic (*1*).

Somalia ranks in the top 15 countries of origin for foreign-born persons with cases of tuberculosis in reported in the United States (*2*). Minnesota has the largest Somali population in the United States (*3*). Although Somali persons constitute <1% of Minnesota's population, they accounted for 30% of tuberculosis cases reported statewide from 1999 through 2003. The unique epidemiologic characteristics of foreign-born tuberculosis patients in Minnesota, and Somali tuberculosis patients in particular, have been described (*1*,*4*).

The emergence of extrapulmonary disease as an important form of active tuberculosis has been noted in many studies (*5*–*7*). Our purpose was to describe the characteristics of extrapulmonary tuberculosis in ethnic Somalis in Minnesota and to assess factors that may contribute to its disproportionately high prevalence in this population.

The Study

Data were obtained from the Minnesota Department of Health's tuberculosis database, specifically, surveillance and public health case management data on all cases of tuberculosis reported among ethnic Somalis in Minnesota from January 1, 1993, through December 31, 2003. Cases were defined in accordance with the Centers for Disease Control and Prevention's surveillance case definition for tuberculosis (*8*).

Of the 407 cases of tuberculosis in ethnic Somalis reported to the Minnesota Department of Health during this 10-year period, 239 (59%) had extrapulmonary involvement, including 198 (49%) with exclusively extrapulmonary disease and 41 (10%) with pulmonary and extrapulmonary tuberculosis. The remaining 168 (41%) patients had pulmonary disease only.

In 2003, 214 cases of tuberculosis (4.4 cases per 100,000 population) were reported in Minnesota; 173 (81%) of these patients were foreign-born, and 58 (27%) were from Somalia. Of the 58 Somali patients, 45 (78%) had extrapulmonary disease. According to US Census data, an estimated 11,164 ethnic Somalis were residing in Minnesota in 2000 (*9*); by June 2004, this population had increased to ≈25,000 (*9*). Based on these numbers, the approximate annual incidence rate of tuberculosis for Somalis in Minnesota in 2003 was 269 cases per 100,000 population, and the approximate rate of extrapulmonary tuberculosis was 209 cases per 100,000 population.

Demographic and clinical characteristics of the 239 Somali patients who had extrapulmonary tuberculosis are summarized in the Table. A total of 179 (75%) patients were tested for HIV within 1 year of the diagnosis of tuberculosis; HIV test results were positive for only 2 (1%).

The only characteristics that differed significantly between patients with extrapulmonary tuberculosis and pulmonary tuberculosis were age and length of time in the United States before diagnosis. Patients who had extrapulmonary tuberculosis were generally older (mean 26.8 years) than those with pulmonary tuberculosis (mean 23.7 years) ($p = 0.01$). Similarly, the length of time between arrival in the United States and diagnosis of tuberculosis was generally longer for patients with extrapulmonary tuberculosis (mean 2.7 years) than for those with pulmonary disease (mean 1.3 years) ($p<0.00001$). In a logistic regression model that controlled for the confounding effects of length of time in the United States on the association between a patient's age and risk for extrapulmonary disease, only the patient's duration of residence in the United States was significantly associated with extrapul-

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Table. Characteristics of 239 Somali patients with extrapulmonary tuberculosis, Minnesota, 1993–2003*

Characteristic	No. (%)
Age, y	
<15	20 (8)
15–24	102 (43)
25–44	99 (41)
45–64	10 (4)
≥65	8 (3)
Sex	
Male	111(46)
Female	128 (54)
Immigration status	
Refugee	178 (74)
Other immigrant	16 (7)
Other/unknown	45 (19)
TST status (n = 210)	
Positive	201 (96)
Negative	9 (4)
HIV status (n = 179)	
Positive	2 (1)
Negative	177 (99)
Culture status, *Mycobacterium tuberculosis* (n = 239)	
Positive	197 (82)
Negative	42 (18)
Drug resistance (n = 197)	
Any first-line drug†	35 (18)
INH	32 (16)
RIF	5 (3)
EMB	4 (2)
PZA	4(2)
MDRTB‡	5 (3)

*TST, tuberculin skin test; INH, isoniazid; RIF, rifampin; EMB, ethambutol; PZA, pyrazinamide; MDRTB, multidrug-resistant tuberculosis.
†INH, RIF, EMB, PZA.
‡Resistant to at least INH and RIF.

monary tuberculosis ($p<0.00001$). Controlling for age, each additional year of residence in the United States was associated with a 30% increase in the risk for extrapulmonary tuberculosis (odds ratio 1.3, 95% confidence interval 1.2–1.5).

A total of 250 sites of extrapulmonary disease were identified in the 239 patients, representing 26 distinct anatomic locations of disease; several patients had disease in multiple sites: lymph nodes (50%), pleura (9%), peritoneum (8%), skin or soft tissue (8%), central nervous system (6%), bones or joints (4%), various organs (miliary) (4%), urogenital tract (2%), and other sites (9%).

Among the 197 (82%) cases of culture-confirmed extrapulmonary tuberculosis, 18% were resistant to ≥1 first-line antituberculosis drug (i.e., isoniazid, rifampin, pyrazinamide, or ethambutol). The prevalence of resistance to specific first-line drugs ranged from 16% for isoniazid to 3% for rifampin and 2% each for pyrazinamide and ethambutol. The prevalence of multidrug-resistant tuberculosis (MDRTB) (i.e., resistant to at least isoniazid and rifampin) was 3%. Resistance to rifampin occurred exclusively with MDRTB (Table).

Of the 186 patients who had extrapulmonary tuberculosis and were treated from 1993 through 2002, 91% successfully completed an adequate course of therapy, 76% within 12 months. Of the 186 treated patients, 55% received strict directly observed therapy, and 25% others received a less intensive form of supervised therapy. Response to treatment did not differ significantly between patients who received directly observed therapy or some other form of supervision and those who administered therapy themselves. One patient died, a 9-year-old girl with MDRTB in multiple sites.

Conclusions

Extrapulmonary tuberculosis is more common than pulmonary tuberculosis in Somalis in Minnesota. Among Somali patients who have extrapulmonary tuberculosis, 50% have lymphatic disease, most are <45 years of age, and slightly more are female; prevalence of HIV infection is low, prevalence of reactive tuberculin skin tests is high, and prevalence of drug-resistant strains is substantial. These findings are similar to those reported for Somali immigrants in other countries (*10*). The prevalence of extrapulmonary tuberculosis among all foreign-born tuberculosis patients in the United States is considerably lower than that reported among Somalis in Minnesota and elsewhere (*11*), which suggests that the unique characteristics of tuberculosis in this population may reflect host factors or differences in geographically endemic strains of *M. tuberculosis*.

In Minnesota, Somali patients who had extrapulmonary tuberculosis were older and had resided in the United States longer than Somali patients who had pulmonary tuberculosis. These differences likely reflect the relative difficulty in diagnosing extrapulmonary disease compared with pulmonary tuberculosis. To minimize the interval between clinical manifestation of disease and diagnosis, clinicians should maintain an increased level of suspicion for extrapulmonary tuberculosis in Somali patients.

Immigration is a major factor in sustaining tuberculosis disease in the United States. This study demonstrates how immigration can affect the local epidemiology of tuberculosis through importation of disease patterns from another part of the world.

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Human Metapneumovirus and Respiratory Syncytial Virus Disease in Children, Yemen

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Factors increasing the severity of respiratory infections in developing countries are poorly described. We report factors associated with severe acute respiratory illness in Yemeni children (266 infected with respiratory syncytial virus and 66 with human metapneumovirus). Age, indoor air pollution, and incomplete vaccinations were risk factors and differed from those in industrialized countries.

Acute respiratory infections (ARIs) are the main cause of childhood death worldwide (*1*). Respiratory syncytial virus (RSV) is most frequently implicated in childhood illness (*2*). Although factors predisposing to severe ARI caused by RSV are well known in industrialized countries, little information exists for developing countries. Infection with human metapneumovirus (HMPV) has clinical symptoms similar to those for RSV (*3*). Despite its frequency, little information exists on factors that predispose children to severe ARI caused by HMPV. We describe factors associated with severe ARI caused by RSV or HMPV in Yemeni children.

The Study

Children ≤2 years of age with ARI attending Al-Sabeen Hospital, a reference hospital in Sana'a, Yemen, were enrolled in a study from October 2002 to May 2003. All children attending emergency and outpatient services between 8:00 a.m. and 1:00 a.m. with signs and symptoms of ARI were recruited independent of disease severity. Diagnosis was based on clinical signs, as suggested by the World Health Organization protocol for research on ARI (*4*). A total of 62% of the patients were hospitalized. Patients admitted at night were recruited the next morning.

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Oxygen pressure (pO_2) was measured by using pulse oximetry (Nonin Medical, Inc., Plymouth, MN, USA), and children were classified as having no or moderate (pO_2 ≥88%) or severe (pO_2<88%) hypoxia, as suggested for high altitudes (*5*), since Sana'a is 2,200 m above sea level. Nasopharyngeal aspirates were tested by using reverse transcription PCR, as previously reported (*6*).

Children with severe hypoxia caused by RSV were compared with children with RSV and no or moderate hypoxia. The same comparisons were used for children infected with HMPV. The χ^2 and Student *t* tests were used, and values with p values <0.20 were entered into backward logistic regressions. Ethical approval was obtained from the ethics committees of the Liverpool School of Tropical Medicine and Al-Sabeen Hospital. Parents were interviewed after informed consent was obtained.

A total of 325 (54%) children were recruited from the emergency service, 235 (39%) from outpatient clinics and 41 (7%) from wards. RSV was identified in 266 (44%) and HMPV in 66 (11%) children, including 25 (4%) coinfected with RSV and HMPV. Two hundred thirty-two (87%) children with RSV, 46 (70%) with HMPV (p<0.01), and 22 (88%) coinfected with both viruses had severe hypoxia (Table). Among RSV-positive specimens, 171 (82%) of 208 were group A and 37 (18%) of 208 were group B. No association was seen between groups or genotypes and hypoxia.

Children ≤3 months of age were more likely to have severe hypoxia if they were infected with both viruses (Figure). Children with severe RSV hypoxia were more likely to have relatives with ARI, and this factor plus a history of recurrent respiratory infections were risk factors for HMPV hypoxia. Personal or family histories of atopy, prematurity, or chronic lung or congenital heart diseases were not associated with ARI severity caused by RSV or HMPV.

Among household characteristics, the presence of animals (cows and donkeys), a person in the house smoking, using private water sources, and cooking with fuels other than gas were more frequent for children with severe RSV hypoxia. Only the presence of cows, private water, and cooking fuels other than gas were associated with severe HMPV hypoxia.

Multivariate analysis showed that age ≤3 months and using cooking fuels other than gas were risk factors for severe RSV hypoxia (adjusted odds ratio [AOR] 3.4, 95% confidence interval [CI] 1.3–8.7 and AOR 10.3, 95% CI 2.2–48) and HMPV hypoxia (AOR 14.2, 95% CI 3.1–65 and AOR 13.1, 95% CI 2.2–78), while incomplete vaccinations (AOR 4.5, 95% CI 1.7–12) and smoking (AOR 3.8, 95% CI 1.5–9.8) were associated with severe RSV hypoxia but not HMPV hypoxia. Conversely, a history of recurrent ARI (AOR 13, 95% CI 2.0–84) was associated with severe HMPV hypoxia but not with RSV hypoxia.

Table. Characteristics of children with mild and severe hypoxia caused by infection with RSV and HMPV*

Characteristic	RSV hypoxia			HMPV hypoxia		
	Mild (n = 34)	Severe (n = 232)	p value	Mild (n = 20)	Severe (n = 46)	p value
Male	22 (65)	154 (66)	0.4	17 (85)	30 (65)	0.08
Mean (SD) age, mo.	7.9 (5.6)	4.2 (4.8)	<0.001	9.1 (5.9)	5.7 (5.4)	0.02
Preterm	1 (3)	11 (5)	0.5	1 (5)	3 (7)	0.6
Height-for-age Z scores ≤–2	13 (38)	73 (32)	0.2	12 (60)	19 (41.3)	0.08
Not exclusively breastfed	12 (35)	136 (59)	0.01	15 (75)	23 (50)	0.1
Incomplete vaccinations	7 (21)	178 (77)	<0.001	4 (20)	30 (65)	<0.001
Recurrent ARI	6 (18)	40 (17)	0.4	2 (10)	15 (33)	0.04
Recurrent wheeze	1 (3)	12 (5)	0.4	0 (0)	5 (11)	0.1
Eczema	1 (3)	11 (5)	0.5	1 (5)	1 (2)	0.5
Asthma	1 (3)	1 (0.4)	0.2	0	3 (7)	0.3
CHD	0	2 (1)	0.7	0	0	0.3
Family member with ARI	18 (53)	165 (71)	0.02	7 (35)	28 (61)	0.03
Family member with allergies	3 (9)	29 (13)	0.3	4 (20)	4 (9)	0.1
Family member with asthma	5 (15)	24 (10)	0.2	1 (5)	5 (11)	0.4
Family member with eczema	3 (9)	11 (5)	0.2	1 (5)	5 (11)	0.4
Smoker at home	9 (27)	120 (52)	0.002	10 (50)	28 (61)	0.2
Indoor animals	9 (27)	97 (42)	0.04	4 (20)	15 (33)	0.2
Cows	1 (3)	52 (22)	0.003	0 (0)	9 (20)	0.02
Goats	6 (18)	69 (30)	0.07	1 (5)	7 (15)	0.2
Chicken	3 (9)	39 (17)	0.1	2 (10)	7 (15)	0.4
Cats	3 (9)	26 (11)	0.4	3 (15)	7 (15)	0.6
Dogs	0	8 (3)	0.3	0	0	0.2
Donkeys	0	32 (10)	0.03	0	4 (9)	0.2
Outdoor animals	2 (6)	88 (38)	<0.001	1 (5)	14 (30)	0.01
Private source of water	11 (32)	138 (60)	0.002	5 (25)	22 (47)	0.04
Cooking fuel other than gas	2 (6)	128 (55)	<0.001	2 (10)	23 (50)	0.001

*Values are no. (%) unless otherwise indicated. RSV, respiratory syncytial virus; HMPV, human metapneumovirus; SD, standard deviation; ARI, acute respiratory illness; CHD, congenital heart disease.

Conclusions

Identification of factors that increase the severity of ARI may affect health policies. However, little information is available about such factors in developing countries. Young children are especially susceptible to severe ARI, and our findings confirm that in a hospital setting age is a factor for both severe RSV and severe HMPV ARI. Prematurity and congenital heart and chronic lung diseases, which have been associated with increased risk for hospitalization for RSV and HMPV infections, were not risk factors. Since most Yemeni children are born at home, with limited access to health services, these children are underrepresented in our sample. We did not identify malnutrition as a risk factor for RSV or HMPV infection. Infection with RSV was more frequent and severe in well-nourished children in Nigeria, The Gambia, and Chile (*7*), but this finding was not confirmed by other investigators (*8*), and it is still contentious.

We found no association of the 2 viral infections with atopy. Although no information exists about their role in infection with HMPV, increased levels of common allergens within households had no effect on RSV infection severity in the United States (*9*). However, Gambian mothers of children hospitalized with RSV infections reported asthma more frequently in their children than mothers of nonhospitalized children (*10*). The role of atopy in development of severe RSV or HMPV infections in developing countries needs further elucidation.

Several reports have suggested that coinfections with RSV and HMPV increase disease severity, but we did not find such an interaction. Although we cannot exclude coinfection with bacterial or other viral agents, for which RSV or HMPV may increase disease severity, our findings are similar to reports from the Mediterranean region (*11*,*12*). This finding suggests that HMPV strains vary with location and time, and certain strains increase disease severity.

Children with severe hypoxia were less likely to be vaccinated, which is a likely indicator of poverty because poor parents in Yemen only use health services if their children are ill and are less likely to have their children vaccinated. In industrialized countries, poverty is associated with a higher incidence of RSV, and risk factors for infection with RSV are more likely to occur simultaneously (*13*).

We also observed that other household characteristics, such as cooking fuel and water, proximity to animals and relatives with ARI, or smoking increased the risk for hypoxia. Although we did not quantify air pollution within households, traditional mud stoves are built at ground level, use wood or dung, and generate large amounts of fumes, which blacken adjacent walls. Exposure to this

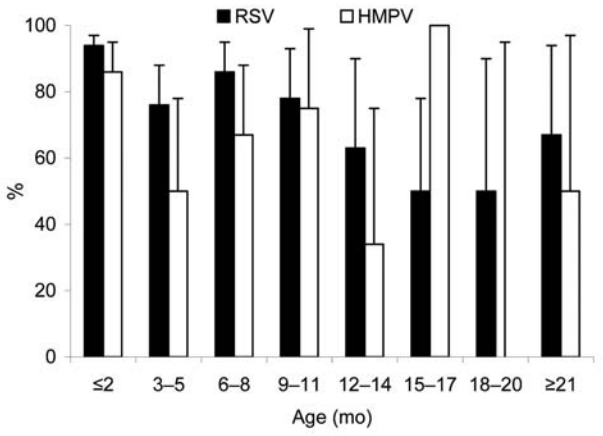

Figure. Proportion of children with severe infection with respiratory syncytial virus (RSV) and human metapneumovirus (HMPV), by age, Yemen. Error bars show 95% confidence intervals.

pollution is associated with severe ARI. Parental and maternal smoking during pregnancy, indoor pollution, and presence of pets are also risk factors in industrialized countries (*14*,*15*). However, in The Gambia the relationship between severe RSV infection and frequency of cooking was inverse (*10*), which may reflect the lower socioeconomic status of mothers who cook frequently; this finding needs to be explored further. Finally, as a hospital-based study, our study had a selection bias toward children with severe ARI, and community-based studies might find different risk factors than those reported here.

In conclusion, age ≤3 months, incomplete vaccinations, persons smoking in the house, and using cooking fuels other than gas were associated with an increased risk for severe RSV hypoxia. Similarly, age ≤3 months, using cooking fuels other than gas, and recurrent ARI were associated with severe HMPV hypoxia. Interventions to eliminate air pollution in households may reduce the severity of RSV and HMPV infections in developing countries, and further studies should be encouraged.

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Hepatitis C Virus Genotype 4 in Ugandan Children and Their Mothers

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In Kampala, Uganda, in 2001, hepatitis C virus antibodies were found in 27 (4%) of 603 children and in 62 (12%) of 525 of their mothers. However, only ≈10% of positive results were confirmed by reverse transcription–PCR, which suggests frequent false-positive results or viral clearance. All sequenced types were genotype 4.

The prevalence of hepatitis C virus (HCV) infection in sub-Saharan African populations is an estimated 3%, with modest regional variation (*1*). The true prevalence may be lower because some positive HCV ELISA results may be due to cross-reactivity, and the reliability of recombinant immunoblot assay (RIBA) as a confirmatory assay is unclear (*2*,*3*). Few studies have confirmed antibody-positive results by molecular methods, and data on HCV genotypes in Africans are limited.

In Uganda, 2 studies showed a low HCV seroprevalence (*2*,*4*), but neither examined samples for viral RNA. In 2001, we conducted a survey of Ugandan children with sickle cell disease and their mothers and found that human herpesvirus 8 infection was associated with transfusion in these children (*5*). Children with sickle cell anemia receive frequent injections during their care, and transfusions are frequently required for anemia. We therefore assumed that our population would be at increased risk for HCV infection. We tested for HCV antibodies and, on antibody-positive samples, sought HCV RNA to confirm antibody reactivity. For RNA-positive samples, we identified genotypes by sequencing and phylogenetic analysis.

The Study

In 2001, we enrolled 603 children (1–16 years of age) attending the Sickle Cell Clinic at Mulago Hospital, Kampala, in our study. By design, approximately half of the children had a history of blood transfusion. When available, the mothers of these children were also enrolled. Participants provided standardized information and blood samples. Plasma and buffy coats were immediately prepared and frozen at −80°C until testing.

Plasma specimens were tested by using an ELISA for HCV antibodies to recombinant antigens c22, c200, and NS5B (ELISA-3.0, Ortho-Clinical Diagnostics, Raritan, NJ, USA) according to the manufacturer's instructions. To conserve samples we did not confirm positive results by RIBA but instead tested for virus in plasma by using a real-time reverse transcription (RT)–PCR assay for HCV RNA developed in our laboratory. This quantitative assay amplified a conserved 155-nucleotide target sequence within the HCV 5′ untranslated region and had an absolute sensitivity of 9 IU/mL of viral load (43 copies/mL). Total RNA was extracted from 140 μL plasma by using the Qiagen Viral RNA Mini kit (Qiagen, Valencia, CA, USA). RT-PCR was performed in a Thermo Hybaid MBS 0.2S (Fisher Scientific International, Hampton, NH, USA) in triplicate reactions that used 10 μL RNA per reaction. After cDNA synthesis, quantitative PCR was performed by using an ABI Prism 7700 or 7900 Sequence Detection System (Applied BioSystems, Foster City, CA, USA). HCV RNA–positive specimens were further characterized by sequencing parts of the Core/E1 and NS5B regions. Briefly, purified RNA was used to generate cDNA by reverse transcription. Nested PCR was performed with sets of published PCR primers to amplify DNA from Core/E1 or NS5B regions (*6*,*7*). The amplification products were separated in an agarose gel and purified by using the Promega DNA purification kit (Promega, Madison, WI, USA). The BigDye Terminator kit (Applied BioSystems) was used to prepare products for sequencing in an Applied BioSystems 310 automated sequencer.

Separate alignments were generated for the NS5B and Core/E1 regions, each including sequences from our samples and genotype 1 to 6 reference sequences obtained from GenBank (*7*). Additional genotype 4 reference sequences used in the analysis are listed in the Figure. These sequences were aligned in ClustalX (version 1.81, Plate-Forme de Bio-Informatique, Illkirch, France) by using the ClustalW (version 1.6, Plate-Forme de Bio-Informatique) matrix and edited in GeneDoc version 2.6 (www.psc.edu/biomed/genedoc). In Mega version 2.1 (www.megasoftware.net), the Clustal alignments for Core/E1 and NS5B were used to generate neighbor-joining trees by using the Kimura 2-parameter plus Γ distribution (K80+Γ) distance model. Free parameters were reduced to the K80 model, and α values were determined by using a maximum likelihood approach in PAUP*4.0 (Sinauer Associates, Inc. Publishers, Sunderland, MA, USA).

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Of 603 children (median age 6.8 years, interquartile range 3.9–10.9 years), 27 (4%) were HCV antibody positive. No significant trends were found between HCV status and demographic (age, sex, tribe) or household (urban/rural location, water supply, room density) variables. Three (5%) of 61 children born to HCV-seropositive mothers were HCV seropositive, compared with 16 (3%) of 456 children born to seronegative mothers (p = 0.58). Eleven (3%) of 322 children with a history of transfusion (mean 1.5 transfusions, range 1–10) were HCV seropositive, compared with 12 (5%) of 245 children with no history of transfusion (p = 0.38). Prevalences in children with (2%) and without (4%) history of scarification were also similar (p = 0.38).

Using RT-PCR to test for HCV RNA, we examined 58 samples from children: all 27 samples with antibody-positive results, 11 with high-negative (near the positive cutoff) results, 10 with negative results from children with a history of frequent transfusions, and 10 with low-negative results from children with no history of transfusion. Only 3 samples, all from seropositive children (ages 6, 7, and 13 years), had positive results by RT-PCR, and viral RNA levels in these samples were 2.3×10^3, 2.8×10^4, and 3.8×10^6 HCV IU/mL plasma.

Of 525 serum samples available from mothers, 62 (12%) were antibody positive. We sought HCV RNA in all 62 mothers with positive ELISA results and in 20 mothers with high-negative results. Five (8%) antibody-positive mothers (ages 28, 30, 38, 39, and 42 years) had RNA-positive samples. Viral levels varied from 6.6×10^3 to 5.7×10^5 HCV IU/mL (median 8.1×10^4). All HCV-seronegative mothers (median age 32.3 years, mean 33.2 years, standard deviation 7.6) were RNA negative.

We amplified samples for sequencing from 5 mothers and 1 child and obtained Core/E1 sequences for all 6 samples and NS5B sequences for 3 samples. None of the sequences was identical to any other or to any sequence in GenBank. Phylogenetic analysis of both the NS5B and Core/E1 regions showed that all viruses amplified clustered within genotype 4 (Figures 1 and 2). In the Core/E1 phylogeny (Figure 2), 2 clusters of HCV strains and 1 outlier (UG1) appeared. Each of the NS5B sequences obtained (Figure 1) was from a different Core/E1 group and they did not cluster. Sequences from this study have been deposited in GenBank (accession nos. AY577578–AY577586).

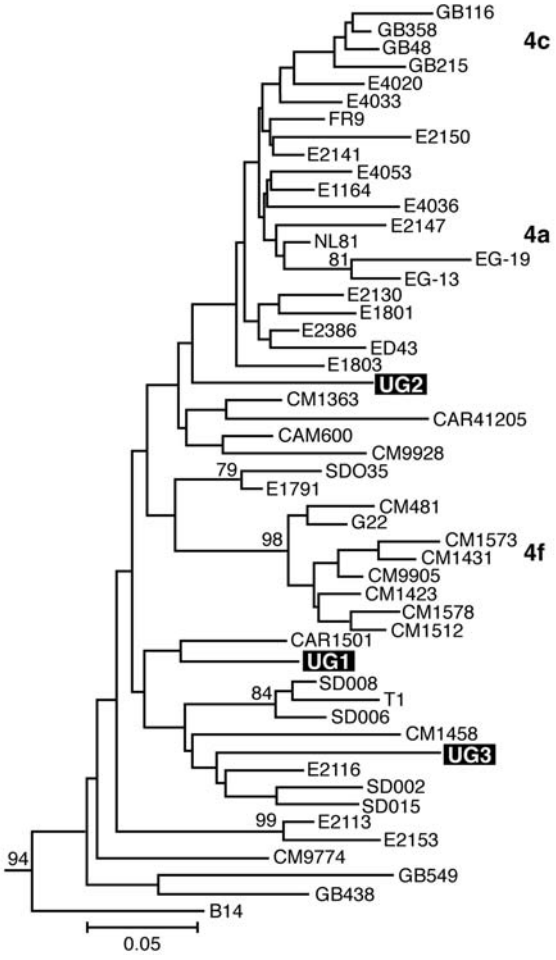

Figure 1. Estimated phylogenies of hepatitis C virus genotype 4; NS5B phylogenetic analysis based on 350 bp of NS5B nucleotide sequence. Ugandan sequences determined in this study are highlighted in black. Numerical values (presented when >60%) represent the statistical support for the tree topology as determined by 1,000 bootstrap replicates. Reference sequences for genotypes 1–3, 5, and 6 (*7*) were included in both analyses and retained as the outgroup. Accession numbers are provided in the text.

Conclusions

We found HCV infection to be uncommon in Uganda, consistent with reports from other Ugandan studies. This cohort of patients with sickle cell disease had frequent blood transfusions, but only 4% of the children were HCV positive by ELISA. Of the seropositive children, only 11% had HCV RNA detectable by RT-PCR. Seropositivity in their mothers was higher, 12%, but only 8% of the seropositive women had positive RT-PCR results. If we assume that untested seronegative patients were not viremic, the overall prevalence of HCV RNA was 0.5% in children and 1% in mothers.

These results might indicate that false-positive ELISA results for HCV are frequent. False-positive results could be due to nonspecific antibody binding or to cross-reactivity with other tropical pathogens, e.g., flaviviruses found in Africa (*2*,*3*). Alternatively, perhaps true antibody-positive participants did not have positive RT-PCR results because they had cleared HCV viremia; however, in the industrialized world, most antibody-positive persons have persistent

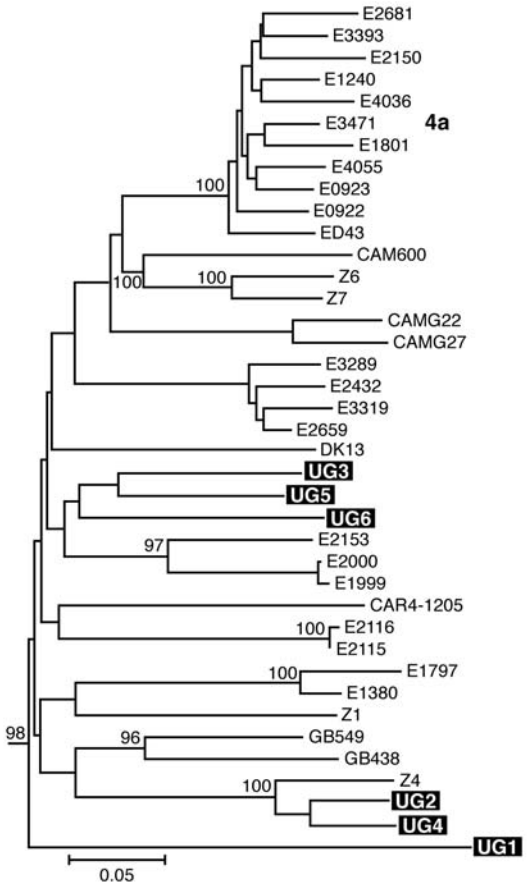

Figure 2. Estimated phylogenies of hepatitis C virus genotype 4; Core/E1 phylogenetic analysis based on 340 bp spanning the junction between the Core and E1 regions. Ugandan sequences determined in this study are highlighted in black. Numerical values (presented when >60%) represent the statistical support for the tree topology as determined by 1,000 bootstrap replicates. Reference sequences for genotypes 1–3, 5, and 6 (*7*) were included in both analyses and retained as the outgroup. Accession numbers are provided in the text.

viremia. We did not confirm ELISA positivity with RIBA because, in Africa, few HCV ELISA-positive samples are confirmed by RIBA (*3*,*8*).

Our RT-PCR results confirm that HCV is present in Uganda, albeit at a low prevalence. How HCV is transmitted in sub-Saharan Africa is not known. We did not find correlations between infection in children and either seropositivity or viremia in their mothers. We expected to see iatrogenic transmission (*1*,*8*) because the children in this study all had sickle cell disease and more than half had had blood transfusions, and many had had multiple transfusions. Most children would have also had potential exposure to HCV from contaminated needles used during the course of their medical care or blades used in scarification by traditional healers (*9*). However, despite these potential exposures, documented HCV infections were uncommon.

Most HCV isolates can be classified into 6 major genotypes on the basis of sequence data from the NS5B, core, and E1 genomic regions. In this study, as in others (*8*), multiple sets of primers were needed to amplify these regions because different primer sets worked for different samples, which reflects the diversity of HCV. Our HCV Core/E1 tree showed 3 clusters. Neither the Core/E1 nor NS5B sequences in Uganda clustered closely with genotype 4 sequences reported from other areas (Figures 1 and 2). Because the number of sequences was limited, we have not assigned subtypes to our newly described variants.

In sub-Saharan Africa, genotype frequencies vary by location (*10*). We found only genotype 4 in Uganda. Genotype 4 has been found in the west-central nations of Africa (Nigeria, Cameroon, Gabon, and Central African Republic), but it also occurs in Tanzania (*11*). Phylogenetic analysis shows that types 1 and 4 share a common ancestor (*12*). Ndjomou et al. (*13*) noted that genotypes 1 and 4 in Cameroon have considerable genetic diversity and suggested that these types may have arisen there. They postulate that genotype 4 spread east into central Africa. Our detection of HCV genotype 4 in Uganda is consistent with their hypothesis, but we do not know the direction of spread. Type 4 is also found in Egypt, where its epidemic distribution is attributed to widespread iatrogenic transmission during schistosomiasis eradication campaigns from 1960 to 1980 (in which needles for injecting potassium antimony tartrate were reused after rudimentary cleaning) (*7*,*14*), and in the Arabian Peninsula (*15*). For many centuries, Arab traders have had extensive contact with African populations of the Nile watershed region, including Uganda, which provides routes for genotype 4 to spread between East Africa and the Middle East.

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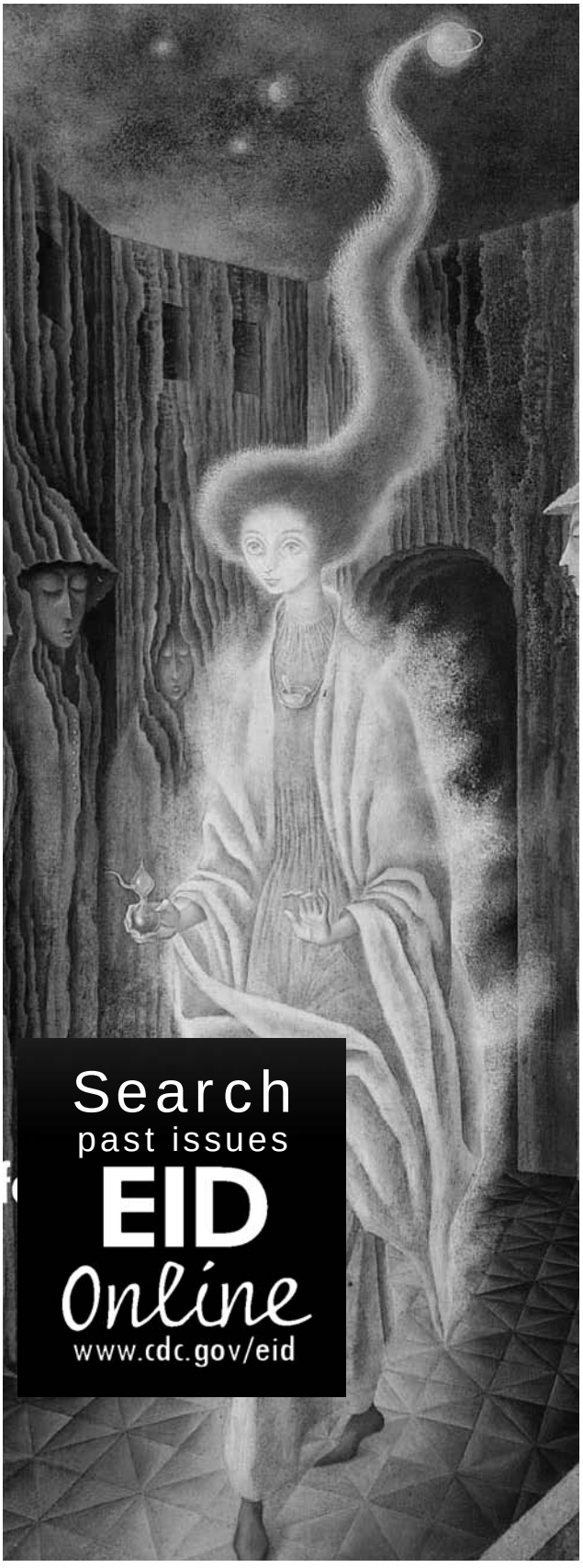

Shift in *Staphylococcus aureus* Clone Linked to an Infected Tattoo

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A retrospective investigation of skin and soft tissue infections caused by community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) strains among inmates in a Wisconsin correctional facility suggested a shift in MRSA genotype. Case timeline indicated a displacement of USA400 clone by USA300 clone. The USA300 index case was associated with an infected new tattoo.

Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is phenotypically and genotypically different from healthcare-associated MRSA (HA-MRSA). Also, risk factors for acquiring CA-MRSA infections differ from those for acquiring HA-MRSA and include crowding, close contact, lack of cleanliness, compromised skin, and contaminated fomites. These risk factors have enabled CA-MRSA to infect persons who meet ≥1 of these criteria.

In the 1990s, outbreaks of CA-MRSA–related infections occurred primarily in certain groups, such as Native Americans, before disseminating into the general population (*1*–*5*). More recently, CA-MRSA outbreaks were seen in unsuspected groups such as military personnel (*6*,*7*), athletes (*8*,*9*), and inmates at large correctional facilities (*10*,*11*). Molecular typing of strains from these recent outbreaks showed that most differed from the predominant clone of the 1990s and belonged to a new CA-MRSA clone, USA300 (*9*). In this retrospective study, we report an outbreak of CA-MRSA–associated skin and soft tissue infections (SSTIs) among inmates of a medium-size correctional facility in Wisconsin. This outbreak was caused by USA400 strains but appeared to be displaced by USA300 clonal group after it was introduced into the facility.

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The Study

This study was approved by the Institutional Review Board of Marshfield Clinic and Marshfield Clinic Research Foundation. Fifteen MRSA isolates were recovered from 15 patients in a correctional facility over a 13-month period (Figure 1). These isolates were recovered from SSTI wound samples submitted to Marshfield Laboratories. The patients were housed in 7 of 10 units with a common recreation yard at a 1,200-inmate facility in Wisconsin from May 2002 to May 2003. Infections with MRSA were rare in this facility; the last reported case of MRSA was 16 months ago. Because of increased number of SSTIs during this period, the Wisconsin Division of Public Health initiated an investigation to determine whether these strains were epidemiologically related.

All strains were typed by pulsed-field gel electrophoresis (PFGE) and staphylococcal cassette chromosome *mec* (SCC*mec*) and tested for virulence genes. Only the first isolate of each PFGE-based clonal group and 1 additional isolate from the same clone were analyzed with *spa* and multilocus sequence typing.

All 15 patients were men (average age 39 years) and had SSTIs at various body sites (Figure 2). All patients, except for inmate Y08, who entered the facility in the seventh month of the outbreak (Figure 1), were incarcerated for 3 to 56 months before the outbreak. Dates of incarceration for 2 inmates could not be determined. All 15 isolates were resistant to β-lactams but sensitive to ciprofloxacin, gentamicin, rifampicin, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin.

PFGE analysis grouped these isolates into 2 clonal groups, USA400 (n = 7, 47%) and USA300 (n = 8, 53%) (Figure 2). The first isolate and a randomly selected second isolate of USA300 and USA400 clonal groups were determined to be sequence type (ST) 8 and ST1, respectively. The representative USA300 strains were *spa* type t008 (YHGFMBQBLO), and USA400 strains were *spa* type t128 (UJJFKBPE). All 15 strains in both clonal groups were type IVa SCC*mec* and positive for virulence factor Panton-Valentine leukocidin (PVL) genes (*lukSF-PV*) and staphylococcal enterotoxin gene *sek*. Isolates of USA400 were also positive for *sea*, *sec*, *seh*, *sel*, and *fnbA*. USA300 strains were positive for *fnbA* and *fnbB*.

Both PFGE profiles (Figure 2) of isolates of USA400 clone were previously observed in Native American communities in Wisconsin throughout the 1990s (*4*). However, ethnicity of the patients in the current study was not determined. PFGE profiles of USA300 strains were indistinguishable from USA300–0114 type strain (*9*). Like USA400 strains, USA300 strains were sensitive to many classes of antimicrobial drugs. However, unlike type 0014 strain, USA300 strains in this study were sensitive to tetracycline. All 8 strains in the USA300 clonal group were

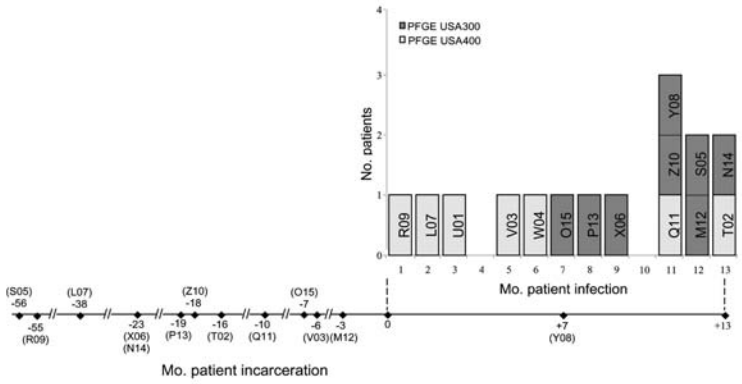


Figure 1. Timeline of incarceration and isolation of methicillin-resistant *Staphylococcus aureus* isolates from different patients. Top panel: baseline shows months in which a particular isolate was recovered and patient was identified as infected; y-axis shows number of patients in each clonal group per month during the outbreak period. Bottom panel: horizontal line shows duration in which patients were incarcerated in relation to outbreak period. Month 0 and month numbers with – and + symbols represent the respective months of incarceration before and after onset of the outbreak, respectively. Codes below months represent patients.

resistant to erythromycin but lacked inducible clindamycin resistance by the D-test (data not shown), whereas only 3 (43%) USA400 isolates were resistant to erythromycin and showed inducible clindamycin resistance.

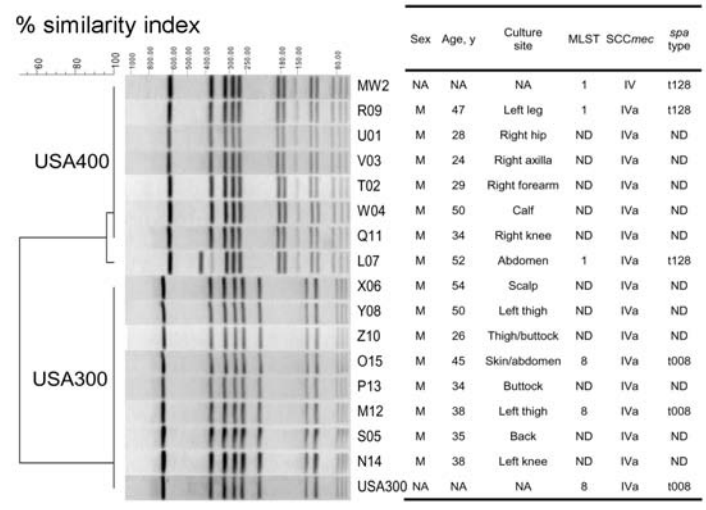
After the outbreak investigation, the facility instituted specific infection control measures for inmates and staff members. Measures included promoting frequent hand washing and improving sanitation of laundry, linens, showers, bathrooms, and equipment in the recreation yard. Inmates were educated about personal hygiene and consequences of sharing needles and other sharp objects. Subsequently, the number of MRSA cases in this facility decreased substantially from 1.25 cases per month during the study period to 0.67 cases per month over the next 6 months.

Conclusions

Molecular typing data for most reported CA-MRSA outbreaks in athletes and prisoners since 2000 showed that these strains belonged to the USA300 clonal group (*9*). This clone has also been reported in the general community in Michigan, predominantly among young African Americans with SSTIs (*12*). Recent reports also indicate that infections with USA300 strains are emerging in neonatal and pediatric groups (*13*,*14*). Therefore, this new CA-MRSA clone is not restricted to groups initially reported (*6–11*) but has reached the community at large (*12–14*).

When and how USA300 clone became established in this Wisconsin correctional facility were not clear. Since interstate transfer of inmates between correctional facilities is common, we speculate that the USA300 clone might have been introduced from such a transfer. The suspected carrier of USA300 clone in this facility could be a colonized inmate (Y08), who was incarcerated in the same month in which the first USA300 strain was identified. The initial case (O15) of the USA300 clone was in an inmate with an infection of a new abdominal tattoo acquired in this facility. The transferred inmate (Y08) was eventually identified as infected with an identical strain in the eleventh month of the outbreak. We speculate that USA300 probably spread among the inmates who were in close contact and shared fomites extensively. Sharing needles and tattoo paraphernalia is common in many correctional facilities. Irrespective of the mechanism of introduction, subsequent SSTIs reported from this facility were mainly due to the newly introduced clone.

USA300 appears to have become the new dominant CA-MRSA clone in a Wisconsin correctional facility, similar to what has occurred in other facilities in the United States. This clonal displacement could be due to better



	Sex	Age, y	Culture site	MLST	SCC*mec*	*spa* type
MW2	NA	NA	NA	1	IV	t128
R09	M	47	Left leg	1	IVa	t128
U01	M	28	Right hip	ND	IVa	ND
V03	M	24	Right axilla	ND	IVa	ND
T02	M	29	Right forearm	ND	IVa	ND
W04	M	50	Calf	ND	IVa	ND
Q11	M	34	Right knee	ND	IVa	ND
L07	M	52	Abdomen	1	IVa	t128
X06	M	54	Scalp	ND	IVa	ND
Y08	M	50	Left thigh	ND	IVa	ND
Z10	M	26	Thigh/buttock	ND	IVa	ND
O15	M	45	Skin/abdomen	8	IVa	t008
P13	M	34	Buttock	ND	IVa	ND
M12	M	38	Left thigh	8	IVa	t008
S05	M	35	Back	ND	IVa	ND
N14	M	38	Left knee	ND	IVa	ND
USA300	NA	NA	NA	8	IVa	t008

Figure 2. Pulsed-field gel electrophoresis (PFGE)-based dendrogram of methicillin-resistant *Staphylococcus aureus* strains isolated during the outbreak. A genetic similarity index scale is shown above the dendrogram. Strain numbers, clone identification, site of infection, and demographic information are included along each PFGE lane. MLST, multilocus sequence typing; SCC*mec*, staphylococcal cassette chromosome *mec*; NA, not available; ND, not determined.

fitness of the USA300 clone than the USA400 clone in vulnerable groups who frequently have >1 risk factor. However, fitness factors that impart advantage to USA300 strains are not clearly identified. Tenover et al. compared genomes of several USA300 strains with USA400 and USA500 and USA100 strains (the last 2 are of healthcare-associated lineages) (*15*). They reported that USA300 strains have several unique sequences in pathogenicity islands such as phi PVL, phi N315, and SaPIn2, in addition to genes encoding several fibronectin-binding proteins such as *fnbA*, *fnbB*, and *ebh*.

Our limited virulence data for USA300 strains in this study showed that they were positive for *fnaA* and *fnaB*, but lacked enterotoxin genes *sea*, *sec*, *she*, and *sel*, some of which are frequently present in the USA400 strains. Genome sequence data from multidrug-resistant USA300 strain FPR3757 showed that it has a novel mobile genetic element that contains genes for enzymes of the arginine deiminase pathway and an oligopeptide permease system (*16*). It is speculated that the arginine catabolic mobile element, which is common in *S. epidermidis* but not in *S. aureus*, probably offers a selective advantage and contributes toward enhanced growth and survival of USA300 on human skin (*16*).

Although risk factors such as close contact, crowded environment, contaminated fomites, lack of cleanliness, and most importantly, compromised skin barriers are crucial in transmitting CA-MRSA–related infections, the role of unknown genomic fitness or virulence factors of USA300 strains cannot be underestimated in its recent spread. Whether certain conditions besides those mentioned also favor establishment of one clone of CA-MRSA over another in the community settings is also not clear (*17*).

We document gradual clonal displacement of USA400 by USA300 in a Wisconsin correctional facility. Sharing of tattoo paraphernalia may be associated with the outbreak and could be considered a possible risk factor for spread of CA-MRSA.

Acknowledgments

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Eighth Major Clade for Hepatitis Delta Virus

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Hepatitis delta virus is the only representative of the *Deltavirus* genus, which consists of 7 differentiated major clades. In this study, an eighth clade was identified from 3 distinct strains. *Deltavirus* genetic variability should be considered for diagnostic purposes. Clinical consequences of the diversity have yet to be evaluated.

Hepatitis delta virus (HDV) is a subviral agent that can lead to severe acute and chronic forms of liver disease in association with hepatitis B virus. Delta hepatitis is highly endemic to several African countries, the Amazonian region, and the Middle East, while its prevalence is low in industrialized countries, except in the Mediterranean. The HDV genome is a circular, single-stranded RNA virus that ranges from 1,672 (strain dFr45, accession number *AX741144*) to 1,697 nucleotides (dFr47, *AX741149*) (*1*). A unique open reading frame encodes the small and large hepatitis delta (sHD and lHD, respectively) antigens by way of an editing step in the hepatocyte nucleus (*2*). Recent extensive analyses of HDV sequences from strains isolated from patients of African origin have shown a high genetic diversity of HDVs. To date, 7 major clades have been individualized with strong phylogenetic support; their proposed labels are HDV-1 to HDV-7 (*1*).

The genetic diversity of HDV is related to the geographic origin of the isolates. Apart from HDV-1, which is ubiquitous, each virus clade is geographically localized: HDV-2 (previously labeled HDV-IIa) is found in Japan (*3*), Taiwan (*4*), and Yakoutia, Russia (*5*); HDV-4 (previously labeled HDV-IIb) in Taiwan (*6*) and Japan (*7*,*8*); HDV-3 in the Amazonian region (*9*); and HDV-5, HDV-6, and HDV-7 in Africa. The eventuality of a genetic diversity extended to more than 7 clades has been mentioned by Radjef et al., who characterized a sequence (dFr644) that was not strongly affiliated to any of the 7 HDV clades (*1*). We describe 2 HDV isolates (dFr2072 and dFr2736) that have robust phylogenetic relation to dFr644 and, therefore, propose an extended classification of the *Deltavirus* genus to 8 clades.

The Study

Strains dFr2072 and dFr2736 were isolated from 2 patients originating from Senegal and Côte d'Ivoire, respectively. The patients were living in France when chronic delta hepatitis was diagnosed; however, because no risk factors were identified, each patient was suspected to have been infected during childhood in Africa.

Full-length HDV genome sequences from isolates dFr2072 and dFr2736 were characterized to determine their genetic affiliation. HDV RNA extraction and cDNA synthesis were performed as previously described (*5*), and 4 overlapping regions of the genome were amplified (Table 1). Amplicons were sequenced bidirectionally with the BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, Courtabœuf, France).

In a first approach, the complete dFr2072 and dFr2736 sequences were aligned with 41 complete genome sequences gathering all of the 7 HDV clades, plus sequence dFr644. The sequence alignment was generated in 2 ways: 1 with ClustalX using a gap-opening penalty (GOP) of 15 and a gap-extension penalty (GEP) of 6.66 and 1 with the SOAP program (http://evol-linux1.ulb.ac.be/ueg/SOAP/) (GOP from 12 to 17 in steps of 0.5; GEP from 6 to 8 in steps of 1). Phylogenetic analyses were performed with PAUP*4.0β10 (Sinauer Associates, Inc., Sunderland, MA, USA) from a SOAP sequence alignment that excluded 833 unstable characters. Neighbor-joining (NJ) distance and maximum parsimony (MP) analyses were performed. The robustness of the topologic features was determined by bootstrap methods (10^3 replicates for NJ and MP). A Bayesian approach (*11*) was also used on the data matrix: 5,000 trees were initially built by using the MrBayes program, version 3.0 β4, from 2×10^6 generations, and the first 250 trees were burned. A majority rule consensus tree was obtained by using PAUP*4.0 β10. Parameters specified during MrBayes analysis (Table 2) were also imported into PAUP*4.0 β10 for a maximum likelihood (ML) analysis using the general time reversible model with a gamma distribution.

An 89.4% similarity score was observed between dFr644, dFr2072, and dFr2736, which allowed these 3 sequences to be grouped inside the same genotype. By contrast, the similarity obtained among dFr644, dFr2072, and dFr2736 and the sequences representative of HDV-1 to HDV-7 was <76.4% (Figure 1). On the phylogenetic tree built from the ML data (Figure 2), isolates dFr644, dFr2072, and dFr2736 appeared as a monophyletic group,

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Table 1. Four overlapping regions amplified by reverse transcription–PCR for full-length genome sequence determination

Region*	Primer name†	Primer position	Nucleotide sequence of the primers (5′–3′)
R0	889s	889–911	CATGCCGACCCGAAGAGGAAAG
(889–1289)	1289as	1289–1265	GAAGGAAAGGCCCTCGAGAACAAGA
R′1	305s	305–328	CTCCAGAGGACCCCTTCAGCGAAC
(305–1161)	1161as	1161–1138	CCCGCGGGTTGGGGATGTGAACCC
R′2	962s	962–984	GTACACTCGAGGAGTGGAAGGCG
(962–331)	331as	331–311	TCTGTTCGCTGAAGGGGTCCT
R′3	120s	120–140	GTCCCAAGAGGGCGAGGGGAG
(120–619)	620as	619–600	TCCTGGAGCCGGCAGTCCGG

*Name of the amplified region and position on the genome (according to Wang et al. [*10*]).
†s, forward primer; as, reverse primer.

Table 2. Parameters specified by MrBayes (version 3.0β4) application

	Substitution rate matrix*						Nucleotide frequencies				
Sequences	G/T	C/T	C/G	A/T	A/G	A/C	pi (A)	pi (C)	pi (G)	pi (T)	α†
Full length	1.000	2.733	0.681	1.344	3.011	0.847	0.200	0.304	0.288	0.208	0.526
sHD‡	1.000	6.687	0.926	2.221	3.550	1.519	0.322	0.216	0.361	0.101	0.434

*Each substitution rate is expressed as compared with the G/T substitution rate.
†Parameter α is the shape parameter of the γ distribution.
‡Small hepatitis delta nucleotide sequence.

with bootstrap values of 100 (NJ and MP) and a posterior probability value of 100 (MrBayes).

Because of claims that the sHD protein trans-complements the corresponding HDV type more efficiently (*12*), we compared the sHD coding nucleotide sequences of dFr644, dFr2072, and dFr2736 with 46 sequences, by using the same phylogenetic approaches (Table 2). Analysis of the sHD genes confirmed the results obtained with the full-length sequences, showing 93.8% similarity between dFr644, dFr2072, and dFr2736 versus only 70.8%–82.9% when compared with sequences of the other genotypes (Figure 1). Bootstrap values of 100 (NJ and MP) and posterior probability values of 100 (MrBayes) were obtained and are represented on the phylogenetic tree built from the ML parameters (Figure 2). Taken together, these results fulfill the recommendations for the designation of a major clade (i.e., ≥3 distinct isolates repeatedly showing high scores of similarity and high bootstrap values [*13*]). Thus, we define an eighth major clade among the *Deltavirus* genus.

Conclusions

In this study, an eighth HDV clade (HDV-8) was identified from 3 complete sequences obtained from strains isolated from patients of African origin. Isolate dFr644, originating from Congo-Brazzaville, was initially described by Radjef et al. and tentatively affiliated with HDV-7 (bootstrap value 84, posterior probability value 97), despite a similarity of only 77.8% with the other HDV-7 sequences (*1*). Isolates dFr2072 and dFr2736 presented similarity of 89.4% with dFr644 and only 76.4% with HDV-7 sequences. Thus, an additional lineage was individualized, bringing the number of HDV clades with a probable African origin to 4.

Since 1999, a total of 468 HDV isolates collected in France were analyzed in our laboratory for phylogenetic characterization of the *R0* region (defined in Table 1). Of these, 98 isolates (21%) were affiliated with HDV-5 (15.2%), HDV-6 (1.7%), HDV-7 (3.0%), or HDV-8 (1.1%) (Paul Dény, unpub data). The 98 corresponding patients were all of African origin. By contrast, all patients of European origin were specifically infected by HDV-1 isolates. To date, no evidence exists that HDV-5, -6, -7 or -8 circulates among native populations in France. These results strongly suggest the African origin of these viruses.

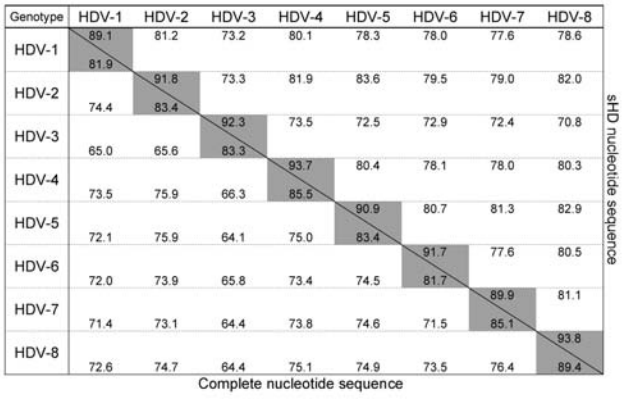

Figure 1. Percent similarity between hepatitis delta virus (HDV) genotypes calculated from complete and small hepatitis delta (sHD) nucleotide sequences. Above the oblique line are represented scores of similarity obtained from alignment and comparison of 49 sHD nucleotide sequences including 13 HDV-1 sequences, 7 HDV-2, 7 HDV-3, 6 HDV-4, 6 HDV-5, 4 HDV-6, 3 HDV-7, and 3 HDV-8. Below the oblique line are represented scores of similarity obtained from alignment and comparison of 44 complete nucleotide sequences including 13 HDV-1 sequences, 7 HDV-2, 4 HDV-3, 6 HDV-4, 6 HDV-5, 3 HDV-6, 2 HDV-7, and 3 HDV-8. Gray cells show the similarities within each genotype.

Nevertheless, epidemiologic studies in Africa should be carried out to specify the prevalence and geographic distribution of all HDV clades. If the African origin of HDV-5, -6, -7 and -8 viruses is confirmed, detection of these clades in France among local populations would reveal an emerging process that should be anticipated in epidemiologic surveys. Thus, the molecular assays used for diagnostic purposes should rely on primers and probes defined in the most conserved regions of the HDV genome to avoid false-negative results (*5*,*14*,*15*).

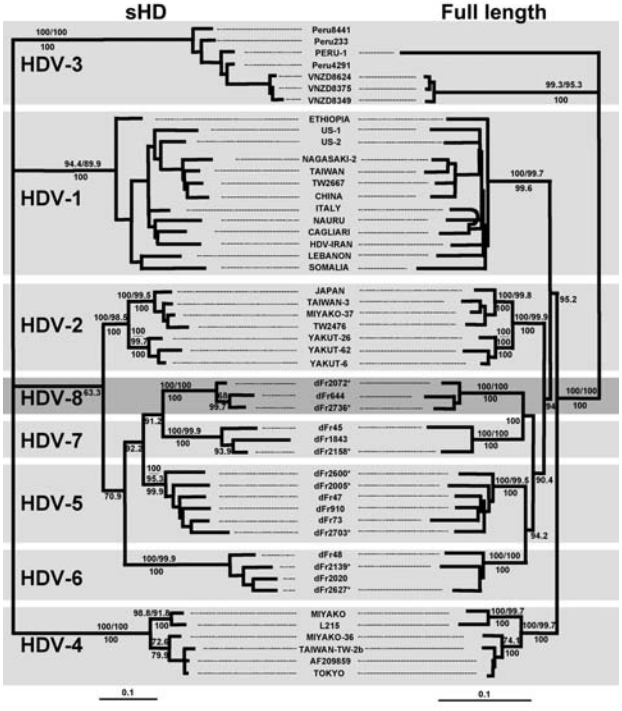

Figure 2. Maximum likelihood trees inferred from hepatitis delta virus (HDV) nucleotide sequences. Left panel: Maximum-likelihood phylogram obtained from the small hepatitis delta antigen dataset. Right panel: Maximum-likelihood phylogram obtained from the full-length HDV genome dataset. Bootstrap values (10^3 replicates) obtained for neighbor-joining and maximum parsimony are indicated above the branches; posterior probabilities (inferred from 5×10^3 trees generated from MrBayes application) are indicated below the branches. Asterisks indicate HDV sequences characterized in this study. Scale is in percent expected substitution per position. The accession numbers of the sequences used were AF209859, *AF209859*; Cagliari, *X85253*; China, *X77627*; dFr45, *AX741164*; dFr47, *AX741149*; dFr48, *AX741164*; dFr73, *AX741154*; dFr644, *AX741169*; dFr910, *AX741159*; dFr1843, *AJ583885*; dFr2005 (Guinea-Bissau), *AM183331*; dFr2020, *AJ583887*; dFr2072 (Senegal), *AM183330*; dFr2139 (Central African Republic), *AM183332*; dFr2158 (Cameroon), *AM183333*; dFr2600 (Togo), *AM183326*; dFr2627 (Nigeria), *AM183329*; dFr2703 (Senegal), *AM183328*; dFr2736 (Côte d'Ivoire), *AM183327*; Ethiopia, *U81989*; HDV-Iran, *AY633627*; Italy, *X04451*; Japan, *X60193*; L215, *AB088679*; Lebanon, *M84917*; Miyako, *AF309420*; Miyako-36, *AB118845*; Miyako-37, *AB118846*; Nagasaki-2, *AB118849*; Nauru, *M58629*; Peru-1, *L22063*; Somalia, *U81988*; Taiwan, *M92448*; Taiwan-3, *U19598*; Taiwan-Tw-2b, *AF018077*; Tokyo, *AB118847*; TW2476, *AF104264*; TW2667, *AF104263*; US-1, *D01075*; US-2, *L22066*; Vnzd8349, *AB037948*; Vnzd8375, *AB037947*; Vnzd8624, *AB037949*; Yakut-26, *AJ309879*; and Yakut-62, *AJ309880*. For larger reproduction of these phylograms, see http://www.cdc.gov/ncidod/EID/vol12 no09/06-0112-G2.htm

In conclusion, the *Deltavirus* genus includes at least 8 major clades, with specific geographic distribution. Future development of molecular assays for diagnosis of delta hepatitis should take into account this high genetic variability. The relationship between HDV diversity and pathogenesis has previously been suggested (*7*,*9*) but remains to be clarified by taking into account the extension of the diversity. Treatment of chronic delta hepatitis, which relies on long-term administration of high doses of interferon-alpha, is not very effective (*16*). It is not known whether some HDV genotypes might be more susceptible to therapy than others, as has been described for chronic hepatitis C (*17*). Thus, the clinical effect of HDV diversity, in terms of severity of disease and response to therapy, remains to be determined.

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West Nile Virus Infection in Commercial Waterfowl Operation, Wisconsin

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A West Nile virus (WNV) outbreak occurred at a commercial waterfowl operation in Wisconsin in 2005. Retrospective analysis of dead and live birds was conducted. WNV was detected by PCR in 84.1% of 88 dead birds; neutralizing antibodies were found in 14 of 30 randomly sampled asymptomatic or recovered birds.

West Nile virus (WNV) is a zoonotic pathogen that cycles naturally between wild birds and mosquitoes. Although hundreds of avian species are susceptible to infection, few instances of disease in commercial flocks of domestic or exotic birds have been reported. WNV infection in domestic geese has been documented in Israel (*1*), Canada (*2*), and Hungary (*3*). These outbreaks were characterized by substantial deaths and high seroprevalence rates. In the United States, an outbreak of WNV associated with high seroprevalence but low death rates was documented in a commercial breeder turkey operation in Wisconsin (*4*). Nonvector transmission was hypothesized to have contributed to the intensity of these outbreaks. We report an outbreak of WNV in a commercial flock of exotic and domestic ducks and geese in Wisconsin in 2005 that was associated with substantial numbers of deaths and a high seroprevalence rate. WNV in this agricultural setting may also have been enhanced by nonvector routes.

The Study

On August 8, 2005, Marshfield Laboratories (Marshfield, WI, USA) was contacted to test 2 deceased captive waterfowl from a farm with a suspected outbreak of WNV. Kidney, spleen, and oral and cloacal swabs were taken and tested by using reverse transcriptase (RT)-PCR (*5*). When tissues and swabs were found positive, we contacted the farm operator to determine the extent and nature of the outbreak and obtained permission to conduct a site visit. Two site visits were made. On the first, on August 18, 2005, we collected frozen, dead carcasses for testing and collected swabs, serum, or both from 8 clinically ill birds. At the second visit, on August 24–25, we collected serum samples.

The farm was primarily operated for production of breeding stock and included >25 species of domestic and exotic species of geese, ducks, and poultry. In a typical year of operation, ≈150 breeding stock are on the farm in early spring; by June the flock expands to ≈1,250 birds. Hatch-year birds are raised to adults and sold to breeders in the fall. An average of 3 deaths per month from various causes, including trauma and infections, occur in the flock. The birds are housed in large, clean, well-drained outdoor pens constructed of wood beams and large-gauge nylon netting. Birds are not segregated by species, and ≤200 birds may be housed together in individual pens. Mosquitoes and small wild birds can move easily through the netting. Each pen contains a concrete pond with continually circulated water that serves as a water source and resting area where the birds congregate each night.

Prior evidence of WNV had been documented on the farm in 2002 but was limited to 5 ducks that died shortly after weakness, tremors, and other neurologic signs developed. At necropsy, WNV was isolated from spleen, kidney, oral swabs, and cloacal swabs from these birds. Infection control interventions used at that time included draining and bleach sterilization of the concrete ponds (J.K. Meece and K.D. Reed, unpub. data).

The farm operator reported that on June 20, 2005, a single Ross goose was noted to have weakness, tremors, head tilt, and drooping wings. This bird died within a day of onset of signs. In the next 2 days, similar neurologic signs developed in 4 more Ross geese, and they died. For several weeks no additional deaths were observed, but from July 22 to July 30, six Siberian red-breasted geese and 2 American widgeons died after displaying neurologic signs. Thereafter, an average of 20 birds of various species died per week until August 17, when the outbreak abruptly ended. During the outbreak, the operator salvaged the birds and stored them at –20°C. Our first visit to the farm occurred on August 18, a day after the last dead bird was collected.

Dead birds (n = 88), saved frozen at –20°C, were returned to the laboratory for testing. The condition of the birds was highly variable; many of the birds had been pecked and partially cannibalized by other flock members. The farm operator identified the American widgeon, Eurasion widgeon, blue-winged teal, and green-winged teal as hatch-year birds. The other species were mixed ages. RNA was extracted from oral-pharyngeal swabs with the RNeasy mini protocol Qiagen kit (Qiagen, Inc.,

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Valencia, CA, USA) and tested for WNV-specific RNA with real-time PCR with the Roche Light Cycler (Roche, Indianapolis, IN, USA) (*5*). Birds that tested negative by oral swabs were necropsied, and their kidney tissues were tested with the same protocol. Overall, 74 (84.1%) of 88 of the dead birds tested were positive for WNV. To assess the sensitivity and specificity of the PCR, diluent from oral swabs from 5 WNV-positive birds and 5 WNV-negative birds were injected onto African green monkey kidney cells (Vero cells, American Type Cell Culture #81-CCL, Manassas, VA, USA) for virus isolation. WNV was recovered in culture from all birds that were PCR positive but not from those that were PCR negative (100% concordance). These culture results were also confirmed by RT-PCR (Table).

Eight live birds that the owner identified as having displayed neurologic signs were captured for sample collection (1 Siberian red-breasted goose, 1 Barrow's goldeneye, 1 blue-winged teal, 2 Eurasian widgeons, 2 Ross geese, and 1 wood duck). Swab samples were obtained from oral and cloacal cavities of these birds. Serum samples, collected from 3 of these birds (1 Siberian red-breasted goose, 1 Barrow's goldeneye, and1 blue-winged teal), showed high antibody titers to WNV. These data are included in the serologic results for cohort B (see below). We detected virus from the oral cavity of 1 of the live, clinically ill Eurasian widgeons.

To assess the extent of WNV exposure to the flock, serum samples from 2 mixed-age cohorts were collected to test for specific antibodies to WNV and Saint Louis encephalitis virus (SLEV) by a constant virus serum dilution neutralization assay during the second site visit (*7*). Cohort A was a group of 58 geese (45 Ross geese, 7 snow geese, and 6 blue geese) that had been removed from the farm at the first sign of bird death and relocated to a site 15 miles away. Cohort B was a group of 12 ducks and 18 geese (9 Siberian red-breasted geese, 2 bar-headed geese, 6 Ross geese, 1 blue goose, 6 Eurasian widgeons, 1 wood duck, 1 redhead duck, 1 Barrow's goldeneye, 2 blue-winged teals, and 1 northern shoveler) housed continuously at the outbreak site; these birds were from the same 3 pens where the bird deaths occurred. WNV-specific antibodies were detected in serum from 5 (8.6%) of 58 birds in cohort A. No antibodies to SLEV were detected in cohort A. All 5 of the seropositive birds were Ross geese; 1 of the 5 was identified as a 2005 hatch-year bird. Antibody titers for cohort A ranged from 10 to 80. WNV-specific antibodies were detected from 14 (46.7%) of 30 birds in cohort B. No antibodies for SLEV were detected in cohort B. Excluding the 3 clinically ill birds (titers below), positive antibody titers were detected in the Siberian red-breasted goose (n = 2), bar-headed goose (n = 1), Eurasian widgeon (n = 5), blue goose (n = 1), Ross goose (n = 1), and blue-winged teal (n = 1). Cohort B was a mixed-age cohort, and we did not determine the age of individual birds in this sampling group. Antibody titers for cohort B ranged from 10 to >320. The farm owner identified 3 birds in this cohort as having been clinically ill. These birds were a Siberian red-breasted goose, a Barrow's goldeneye, and a blue-winged teal with titers of 160, >320, and 160, respectively.

Table. Species of dead birds collected at investigation farm*

Species of dead birds	WNV positive, n (%)	No. WNV negative
Blue-winged teal (*Anas discors*)	16 (100)	0
Northern pintail (*A. acuta*)	4 (100)	0
Green-winged teal (*A. carolinensis*)	3 (100)	0
Falcated teal (*A. falcate*)	3 (100)	0
Northern shoveler (*A. clypeata*)	1 (100)	0
Barrow's goldeneye (*Bucephala islandica*)	1 (100)	0
Cackling goose (*Branta hutchinsii*)	1 (100)	0
Eurasian widgeon (*A. penelope*)†	25 (96.1)	1
American widgeon (*A. americana*)	15 (93.8)	1
Siberian red-breasted goose (*Branta ruficollis*)	3 (50.0)	3
Ross goose (*Chen rossii*)	2 (40.0)	3
Mountain quail (*Oreortyx pictus*)	0	2
Common eider *(Somateria mollissima)*	0	1
Hooded merganser (*Lophodytes cucullatus*) †	0	1
Canvasback (*Aythya valisineria*)†	0	1
Domestic turkey (*Melleagris gallopavo*)	0	1

*WNV, West Nile virus.
†Species reported to Centers for Disease Control and Prevention avian mortality database as having tested positive for WNV from 1999 to present (*6*).

At the onset of the investigation, infections due to avian influenza and exotic Newcastle disease virus were considered in the differential diagnosis. Oral swabs from all dead birds were tested at the Wisconsin Veterinary Diagnostic Laboratory (Madison, WI, USA) for both agents and were negative.

Conclusions

This report is the first to document WNV in a commercial waterfowl operation in the United States. The extent of this outbreak, as evidenced by the seroconversion rate in cohort B, far exceeded deaths in the flock. This outbreak caused a considerable economic loss for the operator, and the occurrence of infection among a large number of birds posed a major occupational hazard to the farm workers.

Our study has limitations because it was a retrospective analysis and we were not able to collect some key data, such as vector infection rates, or to sample the water for WNV. However, the concentrated loss of birds within a small number of housing pens during late July and early August, along with the high seroconversion rate among

asymptomatic and recovered birds in the flock, suggests that nonvector transmission may have occurred. Certain behavior traits of waterfowl may facilitate this phenomenon. Sick birds are regularly pecked and cannibalized by other members of the flock. Previous studies have documented that feather pulp in infected birds often contains high titers of WNV (*8*). In addition, the tendency of waterfowl to congregate on ponds at night provides an opportunity for nonvector transmission through prolonged contact with virus shed into a common water source (*9*). During the mid to late summer, when WNV transmission is highest, most birds in this commercial flock were hatch-year birds and may have been more susceptible to infection (*2*). In contrast, older birds that may have immunity due to prior exposure to WNV made up the minority of the bird population.

Because avian influenza and exotic Newcastle disease were the only other pathogens tested for in this outbreak, we cannot completely rule out the possibility that coinfection with other pathogens contributed to death in certain species. Previous studies have shown that coinfection and other stressors can contribute to high death rates within captive flocks (*3*).

In this outbreak we saw no evidence of symptomatic infection with WNV among the limited number of workers regularly exposed to the birds. In contrast, the outbreak of WNV among breeder turkeys in Wisconsin in 2002 was heralded by illness among farm workers (*4*). In silent outbreaks, the potential risk for humans is masked yet may still be substantial (*10*). In light of growing concerns about a possible avian influenza pandemic, universal precautions, as outlined by the US Department of Labor Occupational Safety and Health Administration (*11*), should be applied when working in avian husbandry. Additionally, more timely reporting of suspected outbreaks to public health officials would permit comprehensive investigations that could elucidate the transmission dynamics of disease in agricultural settings. Timely reporting is also important in implementing control strategies that mitigate spread of infectious diseases to farm workers.

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Dr Meece is a project scientist at Marshfield Clinic Research Foundation. Her current research interests include habitat influences on the transmission dynamics of vectorborne infectious diseases.

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Multidrug-resistant *Mycobacterium tuberculosis*, Bangui, Central African Republic

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We investigated multidrug-resistant (MDR) *Mycobacterium tuberculosis* strains in Bangui, Central African Republic. We found 39.6% with the same spoligotype and synonymous single nucleotide polymorphism in the *mutT1* gene. However, strains had different *rpoB* mutations responsible for rifampin resistance. MDR strains in Bangui may emerge preferentially from a single, MDR-prone family.

Tuberculosis (TB) is a major public health problem and causes 2 million deaths each year. Ninety-five percent of cases are in developing countries, where limited healthcare resources lead to incomplete case and contact tracing, inadequate treatment, and as a consequence, to a larger drug resistance problem (*1*,*2*). Multidrug-resistant TB (MDRTB), defined as resistant to at least rifampin and isoniazid, is more difficult to treat and can cost 100× more than susceptible TB; it is associated with a high death rate in HIV-infected patients (*3*,*4*). MDRTB results from the selection of MDR strains in patients who failed to complete chemotherapy with the correct combination of drugs. The typing of MDR strains can be used to describe transmission and outbreaks, as shown by the identification of MDR epidemics due to the Beijing/W family strains (*5*). Other types, including Haarlem and *Mycobacterium bovis* isolates, have been involved in MDR outbreaks (*6*,*7*). Because MDR strains carry mutations in major metabolic pathways, some researchers have suggested that they may be less virulent and less transmissible (*8*); however, the occurrence of epidemics involving these strains would seem to contradict this suggestion. As with other MDR bacterial species, they may have emerged from strains more adapted to the local population (*9*). We have previously described variations in putative anti-mutator genes in Beijing/W isolates that may have favored adaptive mutations in this family of strains. The failure to show mutator phenotypes in Beijing/W strains suggests that this role may have been transient (*10*,*11*).

We studied MDRTB strains in Bangui, Central African Republic (CAR), because little information has been collected concerning MDRTB in sub-Saharan Africa. In CAR, the incidence of TB is estimated to be 250 per 100,000 inhabitants, and 1.1% of cases are MDR (*12*). In Bangui, ≈15% of the sexually active population is infected with HIV. We spoligotyped MDR strains collected by the Pasteur Institute of Bangui and looked for diversity in a series of putative anti-mutator genes.

The Study

We studied 53 MDR *M. tuberculosis* strains isolated from different patients between 1993 and 2001 at the Bangui Pasteur Institute. Fourteen of these patients were HIV positive, 30 were HIV negative, and 9 were of undetermined status. Epidemiologic enquiries did not show a social link among patients (unpub. data). A non-MDR, nonbiased control group, which included 263 *M. tuberculosis* and 2 *M. bovis* strains, was also studied. These strains included all those from the cohort studied by Espinal et al. (*3*) for which a subculture was obtained.

All 318 isolates were typed by using the spoligotyping method previously described (*13*). Spoligotypes were obtained for 283 (53 MDR and 230 non-MDR strains) of the 318 isolates and were analyzed with BioNumerics software (Applied Maths, Kortrijk, Belgium). Dendrograms were constructed according to degree of similarity (Dice coefficient) and comparison with known spoligotypes. Seventy-nine different spoligotypes were identified: 55 included only a single strain, and 24 included 2–56 strains. The 24 clusters were named A to X (online Appendix Figure, available from http://www.cdc.gov/ncidod/EID/vol12no09/06-0361-appG.htm).

The 53 MDR strains clustered in 8 clusters (Figure, panel A). Twenty-five (47.2%) of 53 clustered in type E, which has characteristics of the T family (ancient *M. tuberculosis* strains with numerous spacers [*14*]). The 230 spoligotype patterns of non-MDR strains were grouped into 22 clusters, and spoligotype E was not a major cluster (Figure, panel B). Cluster Q contained the largest number of strains. Its spoligotype is identical to the DB3 pattern ST47 characteristic of the Haarlem family (*15*). Spoligotypes 97%–99% identical with profiles characteristic of the Haarlem family of strains represent 155 strains. These observations confirm the predominance of the Haarlem type in Africa. However, the Haarlem family was not predominant in our collection of MDR isolates.

The clustering of MDR strains suggested an MDR outbreak; therefore, we looked for other characteristics in

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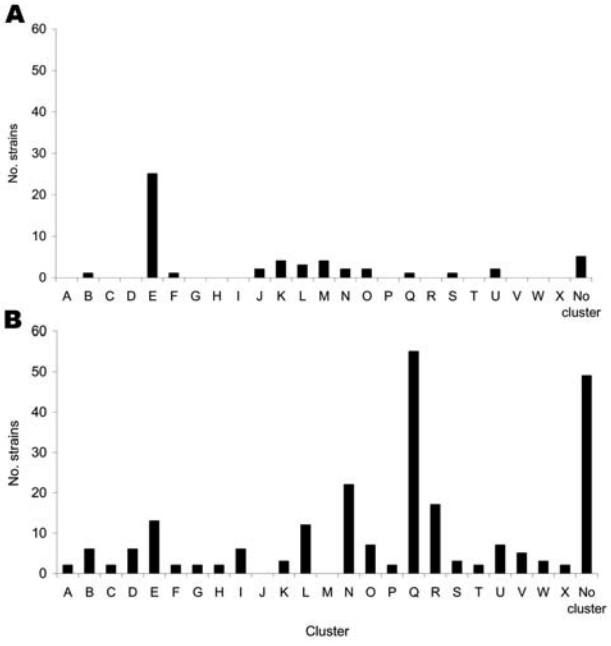

Figure. Strain distribution into various clusters observed among 53 spoligotyped multidrug-resistant (MDR) strains (A) and 230 spoligotyped non-MDR strains (B).

cluster E isolates. First, we tested for diversity in the *rpoB* region, which was likely to be responsible for rifampin resistance. Five variants were found among the 26 MDR strains that constituted clusters E and F (Table): 9 had a Ser-531 (TCG) to Leu (TTG) substitution; 8 and 5 strains contained a substitution of His-526 (CAC) with Tyr (TAC) and Arg (CGC), respectively; 3 had an Asp-516 (GAC) to Val (GTC) variant; and 1 a Leu-533 (CTG) to Pro (CCG) substitution. All these variations are in the rifampin resistance-determining region frequently encountered in strains with a rifampin-resistant phenotype (*8*). These variants probably determine rifampin resistance and may have occurred independently, not necessarily corresponding to MDRTB transmission, even for strains of the same cluster with the same change in *rpoB*. Indeed, sociodemographic and epidemiologic characterization of the patients did not show any links between these MDRTB cases. Therefore, rifampin resistance seems to have been acquired independently and repeatedly by cluster E and F strains. To find a way to reduce the dissemination of such strains likely to generate MDR isolates, we characterized strains of cluster E and F. In particular, we looked for single nucleotide polymorphisms (SNPs) in the putative genes *mutT1*, *mutT2*, *mutT3*, *Rv3908*, *mutY*, *mutM*, *ada/alkA*, and *ogt*. Sequencing was performed as previously described (*11*). With reference to published *M. tuberculosis* sequences, we found 1 synonymous SNP in *mutT1* corresponding to Val 265 (GTC) to Val (GTA). This SNP is only present in strains of cluster E and strains 27 (cluster F), 28, and 29

Table. *rpoB* mutations observed in strains of cluster E and F*

Cluster	No.	ATB	*rpoB*	*mutT1*
E	7	R/I/E	Asp GAC 516 Val GTC	Val GTC 265 Val GTA
E	8	R/I/S	Asp GAC 516 Val GTC	Val GTC 265 Val GTA
E	9	R/I/E/S	Asp GAC 516 Val GTC	Val GTC 265 Val GTA
E	10	R/I/S	His CAC 526 Arg CGC	Val GTC 265 Val GTA
E	11	R/I/E/S	His CAC 526 Arg CGC	Val GTC 265 Val GTA
E	45	R/I/E/S	His CAC 526 Arg CGC	Val GTC 265 Val GTA
E	46	R/I/E/S	His CAC 526 Arg CGC	ND
E	12	R/I/E/S	His CAC 526 Asp GAC	Val GTC 265 Val GTA
E	13	R/I/E	His CAC 526 Tyr TAC	Val GTC 265 Val GTA
E	14	R/I/E/S	His CAC 526 Tyr TAC	Val GTC 265 Val GTA
E	15	R/I/E	His CAC 526 Tyr TAC	Val GTC 265 Val GTA
E	16	R/I/S	His CAC 526 Tyr TAC	Val GTC 265 Val GTA
E	17	R/I/E/S	His CAC 526 Tyr TAC	Val GTC 265 Val GTA
E	18	R/I	His CAC 526 Tyr TAC	Val GTC 265 Val GTA
E	19	R/I/E	His CAC 526 Tyr TAC	Val GTC 265 Val GTA
E	47	R/I/E/S	His CAC 526 Tyr TAC	ND
E	20	R/I/E	Ser TCG 531 Leu TTG	Val GTC 265 Val GTA
E	21	R/I/E/S	Ser TCG 531 Leu TTG	Val GTC 265 Val GTA
E	22	R/I/E/S	Ser TCG 531 Leu TTG	Val GTC 265 Val GTA
E	23	R/I/E/S	Ser TCG 531 Leu TTG	Val GTC 265 Val GTA
E	24	R/I/E	Ser TCG 531 Leu TTG	Val GTC 265 Val GTA
E	44	R/I/E/S	Ser TCG 531 Leu TTG	Val GTC 265 Val GTA
F	27	R/I/S	Ser TCG 531 Leu TTG	Val GTC 265 Val GTA
E	49	R/I/E/S	Ser TCG 531 Leu TTG	Val GTC 265 Val GTA
E	50	R/I/E/S	Ser TCG 531 Leu TTG	ND
E	48	R/I	Leu CTG 533 Pro CCG	ND

*ATB, antibiogram results, indicates strains resistant to rifampin (R), isoniazid (I), ethambutol (E), and streptomycin (S); ND, not determined.

and is absent from all strains in other clusters. Therefore, these MDR strains are characterized by a spoligotype pattern (ST 52 ± spacer 11 or 12 to 15 and ST 107) and the presence of the *mutT1* SNP 265.

MDR strains of clusters E and F and strains 28 and 29 (a single difference in spacers between E and F, strain 28 or 29) corresponded to 9 new cases and 19 patients who had received previous treatment. MDR strains in other clusters corresponded to 9 new cases, 15 previously treated patients, and 1 case for which no history was available (*3*).

Conclusions

We used 2 types of markers to study the genetic diversity of MDR *M. tuberculosis* strains isolated in Bangui: spoligotyping and SNPs in a series of putative DNA repair genes. Many MDR strains were clustered in 1 spoligotype and carried the same SNP in the anti-mutator gene *mutT1*. Indeed, 25 of the 53 MDR strains were in cluster E. Thirty-two percent of these MDR strains were from new cases of infection, and 40% were from HIV-infected patients. This cluster was not a major cluster among the 265 non-MDR isolates collected during a 5-month period. The same SNP was found in all strains of cluster E and F tested and in 2 strains that differed by 1 spacer. These strains carry variants of *rpoB* that confer rifampin resistance, which implies that these strains do not correspond to an MDR-TB outbreak. However, this finding is consistent with the possibility that these strains represent an MDR-prone family, members of which are often associated with MDR phenotypes in Bangui. Detection of strains characterized by the T family spoligotype and *mutT1* SNP 265 may be useful to identify patients at risk of developing MDR-TB.

This article is dedicated to the memory of Eric Kassa-Kelhembo, head of the mycobacterial laboratory in Institut Pasteur of Bangui, who was killed during a mission in CAR within the context of the fight against tuberculosis.

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Dr Nouvel is a molecular biologist and veterinarian. His research interests are in molecular epidemiology of infectious diseases.

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Human Bocavirus in Hospitalized Children, South Africa

To the Editor: In recent years, several novel respiratory viruses have been identified. These include human metapneumovirus (HMPV) (*1*), severe acute respiratory syndrome–associated coronavirus (*2*), human coronavirus (HCoV) NL63 (*3*,*4*), HCoV HKU1 (*5*), and, most recently, human bocavirus (HBoV) (*6*). The latter belongs to the *Parvoviridae* family and is most closely related to bovine parvovirus and canine minute virus (CnMV), which are members of the genus *Bocavirus* (*6*). Parvovirus B19 and HBoV are the only 2 parvoviruses known to be pathogenic to humans, but the relevance of HBoV infection in the clinical setting is not known.

In this retrospective study, 341 nasopharyngeal and bronchoalveolar lavage samples were taken from children (age 2 days–12 years) hospitalized with respiratory tract infections in 2004 in the Red Cross War Memorial Children's Hospital, Cape Town, South Africa. Samples were originally screened by using an indirect immunofluorescence assay (Light Diagnostics, Chemicon International, Temecula, CA, USA) for common respiratory viruses, including respiratory syncytial virus; influenza virus A and B; parainfluenza viruses 1, 2, and 3; adenovirus; and cytomegalovirus. Subsequently, HMPV and HCoV NL63 were detected by using reverse transcription–PCR (*1*,*3*).

Samples were also screened for HBoV DNA. DNA was extracted by using the QIAamp DNA blood mini kit according to the manufacturer's instructions (Qiagen Inc., Valencia, CA, USA). PCR amplification of a region of the NP-1 gene and the 3′ portion of the VP1/2 capsid gene of HBoV was performed. Briefly, 10 μL DNA was added to a 50-μL PCR mix containing 2 IU Supertherm polymerase (JMR Holdings, Kent, UK), 1.5 mmol/L $MgCl_2$, 200 μmol/L each dNTP, and 0.2 μmol/L primers NP-1 s1 (5′-TAACTGCTCCAGCAAGTCCTCCA) and NP-1 as1 (5′-GGAAGCTCTGTGTTGACTGAAT). To improve sensitivity, a second seminested reaction with 2.5 μL outer product and NP-1 as1 primer and NP-1 s2 (5′-CTCACCTGCGAGCTCTGTAAGTA) primer was performed at an annealing temperature of 55°C. Negative controls were used, and appropriate measures were taken to prevent contamination (*7*). Samples with an NP-1–specific PCR product of 368 bp were confirmed by amplifying a 980-bp product of the VP1/2 capsid gene in a similar seminested PCR amplification protocol (primers VP s1 5′-GCACTTCTGTATCAGATGCCTT, VP as1 5′-CGTGGTATGTAGGCGTGTAG, and VP s2 5′-CTTAGAACTGGTGAGAGCACTG). A selection of the inner VP1/2 amplicons obtained from samples taken over the year were sequenced directly and aligned in ClustalX, and a phylogenetic tree was constructed with the Kimura 2-parameter neighbor-joining method with 1,000 bootstrap resamplings. Comparative sequences were obtained from GenBank and included HBoV isolate st1 (DQ000495), HBoV isolate st2 (DQ000496), and a CnMV isolate (NC_004442). Nucleotide sequences from this study were deposited into GenBank (DQ317539–DQ317561).

HBoV DNA was detected in 38 (11%) samples from 35 children, all <2 years of age. Infections occurred throughout the year, although more positive results were found in the autumn/winter season from April to August (63%) than during the rest of the year (37%). A diagnosis of pneumonia or lower respiratory tract infection was made for 30 (86%) children. Thirteen (37%) HBoV-positive children required admission to the intensive care unit. Comorbid conditions were present in 22 children: cystic fibrosis (1), spinal muscular atrophy type 1 (4), Down syndrome (4), cardiac abnormalities (5), and HIV infection (8). Co-infection with a range of viral and bacterial organisms was a common feature in HBoV-positive children and was found in 14 (37%) samples. These organisms included cytomegalovirus (4), respiratory syncytial virus (2), adenovirus (1), HCoV NL63 (1), parainfluenza 3 (1), *Staphylococcus aureus* (1), *Streptococcus pneumoniae* (1), *Klebsiella pneumoniae* (1), and *Pneumocystis jirovecii* (2). However, in the remaining 24 (63%) samples, no other infectious agent was identified.

HBoV was detected in serial samples from 2 children during a 2-day (V04/2591 and V04/2613) and 7-day (V04/2599 and V04/2631) period. In both, sequences were identical and clustered within the proposed subgroup B. In a third child, HBoV sequences were detected in 2 samples taken 2 months apart; in these samples, the isolates were different (V04/1159 and V04/2062) (Figure).

Phylogenetic analysis of the 3′ region of the VP1/2 capsid gene (Figure) showed that the Cape Town strains of HBoV were most closely aligned with the HBoV st2 prototype strain. The nucleotide sequence homology was 98% with 1 amino acid change, N474S. The HBoV st2 branch could be separated into 2 lineages, A and B, with a 3-nucleotide change at positions 4615 (A/G), 4756 (A/C), and 4888 (G/A) on the basis of the numbering of the HBoV st2 sequence.

These results suggest that HBoV infection occurs predominantly during the winter season and that children <2 years of age are most at risk. The study by Sloots et al. (*8*) also found HBoV infections mainly during the winter months (61%) in children <2 years. Although co-infections were found, the proportion (63%) of children in whom only HBoV was detected was

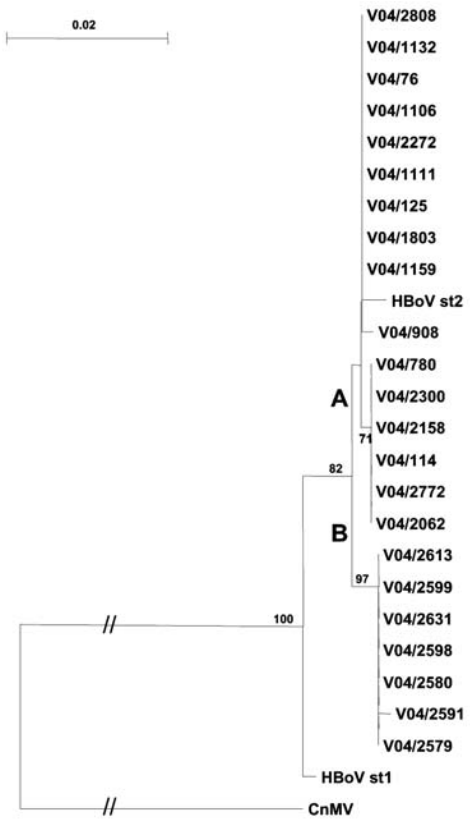

Figure. Phylogenetic analysis of a 980-bp region of the human bocavirus (HBoV) VP1/2 capsid gene from South African children with respiratory tract disease. The tree was constructed by using the neighbor-joining method with 1,000 bootstrap resamplings. All nucleotide sequences were submitted to GenBank (accession nos. DQ317539–DQ317561). CnMV, canine minute virus.

substantial. These findings suggest that HBoV may play a role in respiratory tract infections in young children who require hospitalization.

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Shigella sonnei Outbreak among Homosexual Men, London

To the Editor: In the summer of 2004, genitourinary medicine clinics in London reported cases of *Shigella sonnei* with a novel phage type pattern (later designated PTQ). Outbreak case finding involved local laboratories and genitourinary medicine physicians in London, as well as the national reference laboratory. A case was considered confirmed if *S. sonnei* PTQ was isolated from January 2004 through April 2005, and the patient had not traveled outside the country the week before illness. Possible cases were defined as for confirmed cases but were so designated when patient had a history of foreign travel in the week before illness or when travel history was unknown. From October 2004, when we became aware of the outbreak, until December 2004, we conducted telephone interviews with newly identified case-patients. For cases that occurred before October 2004, and from January 2005 through April 2005, information was obtained from laboratory records only.

Strains were phage typed by using the scheme described by Hammerstrom, Kallings, and Sjoberg, according to a protocol supplied by R. Wollin (*1*,*2*). The scheme consists of 11 phages and is based on the typing of the rough phase II variant of *S. sonnei*. The scheme comprises defined phage types (PT) 1–100 and provisional PTs A–P. Cultures were grown overnight on MacConkey agar, and a rough colony was placed in nutrient broth and grown for 18 hours at 37°C. The broth culture was then used to flood a nutrient agar plate and, once dry, spotted with the 11 phages and incubated at 37°C for 5 hours. The patterns of lysis were recorded and compared with those indicated on the typing chart. All isolates were

screened for resistance to a panel of antimicrobial agents by an agar incorporation method with Iso-Sensitest agar (Oxoid, Basingstoke, UK).

We identified 16 confirmed and 54 possible cases. Specimens from all 70 patients had the same unique pattern of lysis when phage typed, had the same profile when examined by pulsed-field gel electrophoresis, and were resistant to ampicillin, streptomycin, spectinomycin, sulfonamides, tetracyclines, and trimethoprim.

Cases occurred at a low frequency during the first half of 2004, followed by a large increase in August, September, and October (Figure). All case-patients (N = 48) were men, mean age 37 years (range 18–58 years). Five persons designated possible case-patients had traveled abroad in the week before illness (United States, France, Vietnam, Turkey, and 1 unknown destination). Of patients for whom HIV status information was available, nearly all were HIV positive (n = 30/32).

From October 2004 through December 2004, we identified 20 case-patients and interviewed 17 (85%). All were men who had sex with men (MSM). Reported symptoms were diarrhea (n = 15), abdominal pain (n = 14), fever (n = 10), blood in stools (n = 7), and vomiting (n = 6). In the week before illness, 15 reported sex with another man, about half with a casual partner, and mostly with 1 (9/15) or 2 (3/15) different men. No common sex venue was identified. Most (12/15) reported participation in oral and anal sex, and 6 reported oral-anal contact. Three patients recalled that their partner had had diarrhea around the time of sexual intercourse. Of 7 respondents who were asked, 3 reported using a condom during anal intercourse, and none reported using any barrier during oral intercourse.

That all cases were men, and many were HIV-positive MSM, who reported having sex the previous week, strongly suggests that male homosexual sex was the mode of transmission. The shape and timeframe of the epidemic curve indicates person-to-person transmission and rules out foodborne transmission linked to a gay venue. The predominance of HIV-positive homosexual men in the outbreak may be due to more symptomatic disease (from compromised cell-mediated immunity or achlorhydria [*3*]), more unprotected sex with other HIV-positive men (*4*), and greater likelihood of seeking healthcare.

Sexual transmission of shigellosis between MSM was first reported in the United States during the 1970s (*5*), and recent outbreaks have been reported in San Francisco (*6*), Canada (*7*), Australia (*8*), and Germany (*9*). The London outbreak coincided with an outbreak of *S. sonnei* in Berlin, Germany (*10*). Of the 17 Berlin case-patients, 14 were MSM. Isolates from 10 Berlin patients were subsequently tested by the same reference laboratory in London and confirmed to also be PTQ, which suggests a link between these 2 outbreaks, even though none of the London interviewees reported travel to Berlin.

Although the earliest identified case occurred in January 2004, *S. sonnei* PTQ may have been circulating among the MSM community for a longer period. The discovery of an outbreak of a novel phage type underlines the importance of prompt strain-typing for public health investigations and the benefit of good links between local clinicians, laboratories, and public health professionals. Additionally, local gay media and voluntary organizations were valuable partners for disseminating preventative health messages across London when the outbreak was in the early stages. This outbreak raises the possibility that the mobility and increased high-risk sexual practices among MSM in Europe (*4*) might facilitate mixing between sexual networks, thus causing potential for international outbreaks of sexually transmitted infection.

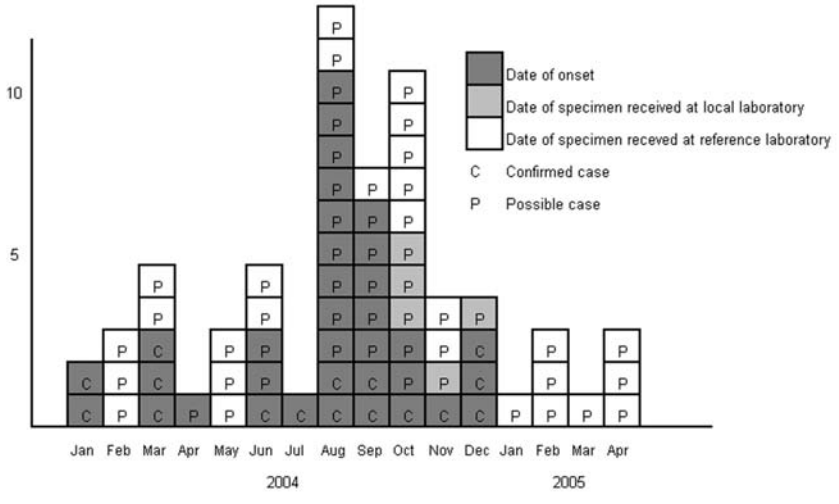

Figure. Confirmed and possible cases of *Shigella sonnei* PTQ by earliest recorded date, London, January 2004–April 2005.

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Perinatal Toxoplasmosis, Northern Taiwan

To the Editor: Toxoplasmosis is caused by a protozoan parasite known as *Toxoplasma gondii*, which is found in animals worldwide and is readily transmitted to humans. The prevalence of *T. gondii*–specific immunoglobulin (IgG) antibodies in women ranges from ≈15% in the United States (*1*) to ≈55% in Europe (*2*). Rate of transmission to a fetus in the first, second, and third trimesters is 8%, 25%, and 60%, respectively (*3*). The rate of congenital toxoplasmosis in the United States is 1–10 per 10,000 live births (*4*). Most infants infected in utero are born without obvious signs of toxoplasmosis, and learning or visual disabilities do not develop in up to 80% until their second or third decade of life (*5,6*).

In 1985 in Taiwan, the prevalence rates of *T. gondii*–specific IgG, as determined by ELISA, for pregnant women and their neonates were 10.2% and 11.6%, respectively. No samples from mothers or neonates were screened for IgM titers (*7*). During the past 20 years, however, the lifestyle, socioeconomic environment, and healthcare system have changed substantially in Taiwan. Overseas traveling has become more convenient, and Taiwan residents often travel to toxoplasmosis-endemic areas. The number of babies born to immigrant mothers has also recently increased in Taiwan. Our objective was to estimate the seroprevalence of perinatally transmitted *T. gondii* in northern Taiwan.

We tested sera collected from consecutive samples of women and their neonates (live births only) at 1 medical center, 1 local hospital, and 2 obstetric clinics in northern Taiwan from April 2004 through January 2005, which was 1 investigation of the Taiwan Birth Panel Study. Informed consent was obtained from either parent before enrollment in the study. Serum samples from cord blood of 483 neonates and paired samples from their mothers were analyzed for *T. gondii*–specific IgG and IgM titers by ELISA (Diagnostic Products Corporation, Los Angeles, CA, USA) (IgG sensitivity 94%, specificity 100%; IgM sensitivity 96.9%, specificity 91%) (*8*). Samples from the mothers were tested within 2 days of delivery. Additional data about health measures and conditions were collected by trained interviewers using structured questionnaires.

Among the study population, 93% were Taiwanese, 0.6% were Taiwanese aboriginals, 2.5% were mainland Chinese, and 3.9% were immigrants from southeastern Asia. Of the 483 mothers, 0.6% worked as farmers, 76% were 25–35 years of age, >50% had a university-level education, 77.7% encountered pets daily, and 9.7% owned cats. Of the 483 infants, the male:female ratio was 50.8:49.2, delivery was premature for 8.6%, and 5.8% had a low birthweight. For the mothers, the *T. gondii*–specific IgG prevalence rate was 9.1% (95% confidence interval [CI] 6.5%–11.7%), and 5 mothers (1.0%; 95% CI 0.1%–1.9%) were IgM positive. The *T. gondii*–specific IgG prevalence rate for the neonates was 9.3% (95% CI 6.7%–11.9%), and 1 neonate (0.2%; 95% CI 0%– 0.6%) was IgM positive (Table).

We identified 2 risk factors for seropositive mothers: being from

Table. Seroprevalence of toxoplasmosis in mothers and their neonates, Taiwan, 2004–2005*

	Mothers		
Neonates	IgG (+)	IgG (–)	Total
IgG (+)	43	2	45
IgG (–)	1	437	438
Total	44	439	483
	IgM (+)	IgM (–)	Total
IgM (+)	–	1	1
IgM (–)	5	477	482
Total	5	478	483

*N = 483; Ig, immunoglobulin.

mainland People's Republic of China (odds ratio [OR] 13.42; 95% CI 1.29–19.49] and being an agricultural worker (OR 59.53; 95% CI 4.45–79.67). Incidence of positive IgG titers was higher for mothers who owned cats than for mothers who did not own cats, but not significantly higher. The neonates who had positive *T. gondii*–specific IgM had negative IgM results at the age of 3 months. No significant differences in gestational age, birthweight, and neurodevelopment were identified between seropositive and seronegative groups of infants.

This study showed that in northern Taiwan, seroprevalence of *T. gondii* among pregnant women and neonates remains low and has not substantially changed during the past 20 years. The reason for such a low incidence in Taiwan is not clear but may be attributed to differences in the lifestyle, climate, cultural differences, food habits, and to the lesser consumption of raw meat in Taiwan compared with western countries. We found that the rate for *T. gondii*–specific IgM is higher for mainland Chinese mothers than for Taiwanese mothers ($p<0.05$). In Taiwan, the percentage of immigrant mothers increased from 15.7% in 1998 to 32.1% in 2003, and the percentage of neonates born to these mothers increased from 5.1% in 1998 to 13.8% in 2004 (*9*). Whether the lifestyle of mothers in Taiwan with a different ethnicity will influence future *T. gondii* seroprevalence require further investigation. This study highlights the emerging importance of toxoplasmosis as a possible perinatal infection in Taiwan. A high index of suspicion for infectious diseases among immigrant mothers is needed (*10*).

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Fluoroquinolone-resistant *Streptococcus pneumoniae*

To the Editor: In pneumococci, resistance to fluoroquinolones is associated with chromosomal mutations in the quinolone-resistance–determining regions (QRDR) of type II topoisomerase enzymes, predominantly *gyrA* and *parC*. Several mutations have been described in these enzymes, but only a few have been shown by in vitro studies to confer resistance: S81F or Y, C, or I and E85K in *gyrA*; E474K in *gyrB*; A63T, S79F or Y or L and D83G or N in *parC*; and E474K and D435N or H in *parE* (*1–5*). Other frequently described mutations are K137N in *parC* and I460V in *parE*, which appear to not contribute to fluoroquinolone resistance because they are commonly found in susceptible strains, and no evidence exists for their conferring fluoroquinolone resistance in vitro. We describe here a pneumococcal strain that was isolated from a 66-year-old white man with chronic obstructive pulmonary disease (COPD).

The patient was admitted to the hospital with a presumed exacerbation of COPD. He had been discharged from the hospital 2 days earlier, having recovered from a similar manifestation of this disease. His treatment history was 250 mg/day oral levofloxacin for 7 days while in the hospital and levofloxacin for 10 days as an outpatient for a similar lower respiratory tract infection 3 months earlier.

On this second admission he was given levofloxacin, 250 mg intravenously, once a day. He was treated with a low dosage because he was in renal failure. The patient continued to worsen and was transferred to the intensive care unit, where ceftriaxone, 1 g intravenously once a day, was given along with levofloxacin. He improved on the combination therapy and was discharged without sequelae.

Cultures of the patient's blood and sputum grew *Streptococcus pneumoniae*. The isolate from blood was resistant to levofloxacin (MIC 8 mg/L) and ciprofloxacin (MIC 8 mg/L), yet susceptible to gatifloxacin (MIC 1 mg/L) and ceftriaxone (MIC 0.38 mg/L), with intermediate resistance to penicillin (MIC 1.5 mg/L). The resistant isolate was of serotype 6A and of multilocus sequence type 376, which is the North Carolina^{6A}-23 clone (http://www.sph.emory.edu/PMEN/index.html).

Efflux testing that compared the ciprofloxacin MICs in the presence and absence of reserpine (10mg/L) showed no evidence of an overexpressed efflux pump. We sequenced the QRDRs (*gyrA*, *gyrB*, *parC*, *parE*) and the entire *gyrA* and *parC* genes of the resistant strain isolated from blood by using previously described primers (*2*). Sequencing showed a S79Y mutation in *par*C and a Q118K (CAA→AAA) mutation in *gyr*A. Sequencing of the entire *gyrA* and *parC* genes confirmed that no additional amino acid substitutions were outside the QRDRs. The entire *gyr*A gene PCR product was transformed directly into the susceptible pneumococcal reference strain R6 by a standard transformation protocol (*4*). Transformants were selected on plates containing increasing concentrations of ciprofloxacin and, in a second step, were transformed with the entire *parC* gene of the resistant strain.

The ciprofloxacin and levofloxacin MICs of R6 transformed with the *gyrA* gene of the resistant isolate containing the new Q118K mutation were 4 and 2 mg/L, respectively. After additional transformation of these transformants with *parC* of the resistant isolate containing the S79Y mutation, the selected double transformants exhibited the same MICs as the original clinical isolate (8 mg/L for ciprofloxacin and levofloxacin). The transformation of *parC* alone conferred an intermediate increase in the MICs (ciprofloxacin 2 mg/L, levofloxacin 4 mg/L). All transformants were confirmed by sequencing.

To determine the biologic cost associated with the different resistance mutations in vitro, each fluoroquinolone-resistant mutant was competed against the fluoroquinolone-susceptible parent strain R6 (with an independent streptomycin resistance marker) as described by Johnson et al. (*6*). The outcome was evaluated as the change in the ratios of the competing strains as a function of the number of generations. Each competition was performed in triplicate by using independent starting cultures of each competing strain. Compared with the wild-type R6 strain, the relative fitness values for the *gyrA*, *parC*, and double mutants were 1.06, 1.03, and 0.93, respectively.

These data indicate that a single mutation in either *parC* or *gyrA* does not impose a substantial fitness burden. In contrast, the double-mutation *parC* S79Y and *gyrA* Q118K was associated with a slower growth rate. Similar results of relative fitness for single (*parC* S79Y and *gyrA* S81F) and double mutations were observed by Gillespie et al. (*7*).

Development of resistance to fluoroquinolones is a stepwise process, involving spontaneous mutations in the genes encoding the target enzymes DNA gyrase and the topoisomerase IV. Mutants with mutations in 1 of the enzymes are estimated to arise at a frequency of 1 to 10^{-7} (*1*). Therefore, fluoroquinolone resistance due to selection of spontaneous mutants during treatment may be related to the number of bacterial cells in the population under selective pressure. Patients with COPD are frequently colonized by high bacterial loads. COPD has been identified in several recent studies as an independent risk factor for fluoroquinolone resistance (*8*,*9*). Low doses of fluoroquinolones

may also lead to an increased risk for resistance selection (*10*). Because the Q118K mutation has not been previously described, this new mutation was probably selected by the current or antecedent treatments rather than by an infection with a resistant widely disseminated clone.

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Spring Scrub Typhus, People's Republic of China

To the Editor: Pingtan Island, in the eastern Fujian Province, People's Republic of China, has been a traditional focus of summer scrub typhus. In the early 1950s, the health of the residents was compromised by scrub typhus, with an incidence of 1,000 cases/100,000 population and a case-fatality rate of 13.6%. With the understanding of the pathogen and application of effective treatments (*1*,*2*), the epidemic was brought under control. Documentation showed that from 1960 through 1990, the annual incidence of scrub typhus maintained a level of 50–100 cases/year. Since 1990, cases have decreased sharply.

The usual epidemic season for scrub typhus on the island was summer. The first outbreak of spring scrub typhus occurred in 2000 in the town of Beicuo; 10 cases were reported. Beicuo, population 10,000, is located in southwestern Pingtan Island. The first patient visited the local hospital on April 6, 2000, with a high fever, cough, and headache. The initial exclusion of scrub typhus because of the spring time frame made the diagnosis difficult until a typical black eschar was found on the patient's waist. This case called attention to spring scrub typhus and led to the timely diagnosis and treatment of the subsequent cases. By 2005, a total of 28 spring cases were on file. An indirect immunofluorescence antibody method with Gilliam strain antigen, as described (*3*,*4*), was applied to the above samples for serologic analysis. Samples with antibody titers ≥64 were considered diagnostic. All 28 case-patients were identified as having antibodies to *Orientia tsutsugamushi* 8–20 days after the onset of the disease (Table).

The number of cases of spring scrub typhus from 2000 to 2005 were

Table. Clinical manifestations of spring scrub typhus, 2000–2005, Pingtan Island, People's Republic of China

Characteristic	No. patients (%)
Male	24 (86)
Female	4 (14)
Symptom	
Fever	28 (100)
Eschar	25 (89.2)
Node	28 (100)
Rash	25 (89)
Cough	19 (68)
Headache	13 (46)
Chill	15 (53)
Fatigue	16 (57)
Myalgia	10 (35)
Vomiting	8 (29)
Serologic titer	
IgG titer of IFA,* median (range)	1,280 (320–2,560)
Age, y	
Median (range)	21.5 (7–59)

*IFA, indirect immunofluorescence assay; IgG, immunoglobulin G.

10, 7, 9, 0, 0, and 2, respectively. The disease was prominent in farm workers aged 40 to 49 years (10 cases). Most younger persons aged 20–40 years, had left the area for better income, so their case number was relatively low (4 cases). Five patients were military personnel, of which 4 were susceptible new recruits from various regions where scrub typhus was not found. Eight cases were associated with children who often played in the grassland and woods. The chance of getting infected with the scrub typhus agent is increased by frequent exposure to the vector mites, which inhabit areas rich in vegetation.

We performed an investigation on the possible hosts and vectors of spring scrub typhus since 2002. Rodents were trapped in April and May 2002. Of 246 captured rodents, *Rattus losea* comprised 32.5% of the collection and had a high mite-carrying rate and mite-carrying index (87.5% and 19.9%, respectively). Mites were collected from the captured rodents. Among these mites, 2,100 *Leptotrombidium deliense* accounted for 94.1% of all the mites.

O. tsutsugamushi was isolated by peritoneally injecting mice of KM species with the patient's untreated blood, the triturated viscera of the rats (*R. losea*), and the triturated mites (*L. deliense*). This process resulted in 3 identification of *O. tsutsugamushi* strains, which we named Ptan, Ptan2, and Ptan3 (GenBank accession nos. DQ517961, DQ517962, and DQ517963). PCR was performed as previously described (*5*), and the sequences of the gene encoding the 56-kDa protein from the 3 *O. tsutsugamushi* isolates shared >99.8% homology. They also shared 96% homology with *O. tsutsugamushi* Karp strain.

This study verified Pingtan Island as a focus of spring scrub typhus by demonstrating the existence of the pathogen among patients, a rodent host *R. losea*, and the vector *L. deliense*. As demonstrated by Yu et al. in 1953 (*6*), *R. losea* and *L. delinese* were also the host and vector of summer scrub typhus on the island. *L. delinese* formerly appeared in late May and now can be found in March. The earlier appearance of these mites might be related to the warming weather. The local meteorologic data showed that between 1953 and 1996, the average March temperature never exceeded 12.7ºC. However, since 1997, temperature increases have been recorded. During 2000–2002, the average March temperatures were 13.8°C, 15.1°C, and 16.4°C, for each year, respectively. The earlier appearance of the vector mites might explain the spring cases of scrub typhus. No cases of scrub typhus were reported in 2003 and 2004. This finding might be due to the successful preventive measures, including education about scrub typhus and instructions for using personal protective gear against mite bites when working in the fields. Also in 2003, an unusually low amount of precipitation limited the growth of vegetation, which subsequently restricted the habitat of the mites.

When we compared the patients with spring and summer scrub typhus, we observed similar epidemiologic characteristics, including clinical symptoms, pathogen hosts and vectors, and epidemic pathway. The local meteorologic records confirmed an increase in average March temperature since 1997. We suspect that these reports of spring cases represent a widening of the epidemic season of summer scrub typhus because of the increase in local temperature. We plan to seek confirmation by comparing the genetic relatedness of this spring scrub typhus isolate with that of the summer isolate, serologically identified by Yu et al. (*7*) as Gilliam type.

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Early Neuroschistosomiasis Complicating Katayama Syndrome

To the Editor: Neurologic complications of schistosomiasis may occur early as well as late in the course of infection; they result when a pair of worms becomes lodged in the vasculature, and their eggs become trapped in the microcirculation of the brain or spinal cord. There, they elicit a strong inflammatory response, which causes the clinical manifestations (*1*–*3*). Magnetic resonance and computed tomographic images of the brain show nonspecific, contrast-enhancing infiltrates, which suggests brain tumors (*4*). Definitive diagnosis requires finding *Schistosoma* eggs in feces, urine, rectal biopsy specimen, or biopsy specimen of central nervous system lesions (*5*), while a positive antibody test result provides a probable diagnosis only. To prevent irreversible damage, early treatment with corticosteroids is essential, after which the adult worms can be eliminated with praziquantel (*3*,*4*). A high degree of suspicion is therefore needed to avoid treatment delay.

Neuroschistosomiasis has been reported in persons living near Lake Malawi, in Malawi (*6*). Four members of a Belgian expatriate family (both parents and 2 children, a 12-year-old boy and a 7-year-old girl) went swimming in Lake Malawi in September 1998. On the advice of a physician, they took praziquantel 2 weeks afterward as postexposure prophylaxis. Nevertheless, fever, hypereosinophilia, cough, and abdominal discomfort developed in the mother and both children 6–8 weeks after they had been swimming; these symptoms were indicative of Katayama syndrome. The father remained asymptomatic but had a moderately raised eosinophil count (760 cells/mm^3) and tested positive for *Schistosoma* antibodies. *Schistosoma hematobium* eggs were found in feces and urine of the mother and girl. All family members tested negative for schistosomiasis on a screening visit the previous year. The boy was admitted to a Zambian hospital because of high fever, cough, and a pulmonary infiltrate. He did not improve on antimicrobial drugs given for suspected pneumonia, and a gradually worsening neurologic syndrome developed, with left-sided hemiparesis, slurred speech, and slow movements.

The boy's condition prompted repatriation ≈10 weeks after the exposure. On admission at the University Hospital of Antwerp, his symptoms included fever, left-sided paresis with left-sided Babinski sign, and high eosinophil count (3,080 cells/mm^3). An ELISA for *Schistosoma* antibodies was weakly positive. Examination of spinal fluid showed normal cell and protein content and a slightly lowered glycorrachia. A nuclear magnetic resonance (NMR) image of the brain showed multiple, small, contrast-enhanced white matter lesions around the semiovale center (cranially from the lateral ventricles) bilaterally and in the right parietal cortex. A tentative diagnosis of acute neuroschistosomiasis was made, and the patient was given corticosteroids with praziquantel, 750 mg twice a day for 14 days. At the end of this treatment, his condition had markedly improved; discrete hemiparesis was the only residual symptom. One month later, the patient had returned to normal, apart from left leg hyperreflexia. An NMR of the brain still showed residual lesions around the semiovale centers. Ten months later, results of clinical and neurologic examinations were normal, but NMR of the brain still showed minor residual lesions around the semiovale center on the right side. During follow-up, a serologic shift (indirect hemagglutination schistosomal antibody test) was seen, and eosinophil count decreased gradually to normal (Table). Although the boy never excreted eggs, *S. hematobium* infection was presumptively diagnosed on the basis of active infection in his relatives and the response to treatment.

When neurologic symptoms appear soon after primary infection with *Schistosoma* flukes, confirming the diagnosis may prove difficult, and schistosomiasis should be suspected when the patient has bathed in poten-

Table. Evolution of acute neuroschistosomiasis in 12-year-old boy after treatment

Parameter	Day 0	Day 45	Month 10
Neurologic symptoms	Present	Present but diminished	Absent
Eosinophil count (per mm^3)	3,080	1,030	370
Schistosoma ELISA	Weakly positive	Weakly positive	Negative
Schistosoma indirect hemagglutination assay (antibody titer)	Negative	640	80
Urine microscopic analysis	Normal	Not available	Normal
Urine concentration test for schistosomal eggs	Not available	Not available	Negative
Feces concentration test for schistosomal eggs	Not available	Not available	Negative

tially infected water. Furthermore, hypereosinophilia is an early warning sign, as seroconversion and egg excretion may be slower to evolve. Both elements provide sufficient circumstantial evidence to strongly suspect the diagnosis (*2*). In this case, the full-blown Katayama syndrome contributed to the evidence.

Praziquantel only kills adult worms and does not inactivate schistosomules, nor the miracidium inside the eggs, which will continue to elicit a damaging immunologic response for some time. Early antischistosomal treatment might, in fact, worsen symptoms (*7*). Because schistosomules may require up to 8 weeks to mature, early postexposure treatment with praziquantel cannot be used to forestall disease after primary infection. Furthermore, Katayama syndrome may occur as early as 3 weeks after exposure. On the other hand, withholding praziquantel until larvae have matured (8 weeks after exposure) would not prevent Katayama syndrome in many cases (*7*). Acute symptoms, including early neuroschistosomiasis, may therefore still develop during this 5-week window after exposure, despite early praziquantel administration.

Artemether has shown promising activity against schistosomules (*8*). Repeated administration throughout the transmission season has prevented Katayama syndrome in *S. japonicum* infection (*9*). Its use, singly or in combination with praziquantel, should be investigated as true postexposure prophylaxis for primary schistosomal infection in nonimmune travelers (*10*).

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Murine Typhus from Vietnam, Imported into Japan

To the Editor: In Vietnam, many febrile diseases such as malaria, dengue fever, Japanese encephalitis, scrub typhus, and more recently, severe acute respiratory syndrome (SARS) and avian influenza have been reported. Murine typhus cases were also reported during and before the 1960s but not thereafter (*1–5*).

On May 3, 2003, a 54-year-old male resident of Tokushima, Japan, had onset of fever in the suburban town of Cu Chi, ≈60 km northwest of Ho Chi Minh City, Vietnam. Exanthema appeared on his trunk and limbs on May 7. He returned to Japan on May 9 and was admitted to

Tokushima University Hospital on May 10. His body temperature was 39.0°C, and serum, C-reactive protein level was high (17.06 mg/dL) on admission (day 8 of illness). Unfortunately, the blood sample taken on that day was discarded. We then collected blood on days 10, 11, 12, 14, 17, and 24 of illness for diagnosis. Minocycline was administered on day 8 and resulted in a gradual decrease in fever and rash. Weil-Felix tests on day 12 showed the serum to be positive for *Proteus vulgaris* OX19 (titer 160); tests for *P. vulgaris* OX2 and OXK were negative (titer of 10 for both). We examined blood samples for possible diseases such as malaria, dengue fever, SARS, and rickettsioses. Giemsa-stained peripheral blood samples obtained on day 11 showed no malarial parasites. Results of immunoglobulin M (IgM)-capture ELISA of serum on days 10, 11, and 17 of illness were negative for dengue antibodies. Reverse transcription (RT)–PCR of the serum on day 11 was also negative. RT-PCRs of a pharyngeal swab and urine collected on day 11 were both negative for the SARS coronavirus. These specimens were also injected into Vero cells, and no cytopathic effects were generated. RT-PCR of these cultures was also negative for SARS coronavirus. Moreover, SARS antibodies were not found in serum samples on days 11 and 14 of illness. Serum was also tested for *Orientia tsutsugamushi* and *Coxiella burnttii* on day 12 to exclude scrub typhus and Q fever as diagnoses.

Indirect immunofluorescence tests for etiologic agents of spotted fever, murine typhus, and epidemic typhus were then performed with serum samples collected on days 10, 14, and 24. We used *Rickettsia typhi* and *R. prowazekii* as typhus group (TG) rickettsial antigens and *R. japonica* and *R. conorii* as spotted fever group (SFG) rickettsiae. IgM antibody was detected for these antigens, indicating that the disease was a primary infection of rickettsiae (Table). When TG and SFG rickettsioses were compared, TG rickettsiae represented markedly higher elevated titers than SFG rickettsiae, which excluded a diagnosis of SFG rickettsiosis. PCR for the TG rickettsial genome in the convalescent-phase serum on day 10 was negative.

To demonstrate more detailed antigenic reactivity, Western immunoblotting was performed with serum on day 14 (*6*). The serum reacted similarly to the ladderlike lipopolysaccharide (LPS) of *R. typhi* and *R. prowazekii*. As expected from the group-specific nature of rickettsial LPS, no reaction was demonstrated to LPS of SFG rickettsiae, *R. japonica* and *R. conorii*, although weak reactivity, mainly to the major outer member protein of SFG rickettiae, rOmpB, and molecules of smaller sizes was shown (*6*,*7*). As described previously, rOmpB has cross-reactive antigenicity between TG and SFG rickettsiae (*6*). Compared with the trace reaction to rOmpB of SFG rickettsiae, an extremely high level of reaction was demonstrated to rOmpB of TG rickettsiae. These results confirmed the disease to be a TG rickettsiosis.

To elucidate whether the disease was murine typhus or epidemic typhus, we conducted cross-absorption tests as described previously (*8*,*9*). Serum absorbed by *R. typhi* showed complete absorption, demonstrating no reaction to *R. typhi* or *R. prowazekii* (Table). However, the serum absorbed by *R. prowazekii* resulted in incomplete absorption, demonstrating no reactivity to *R. prowazekii* but some reactivity to *R. typhi*, which was left unabsorbed. Western immunoblotting with the serum absorbed by *R. prowazekii* showed reactivity only to the rOmpB of *R. typhi* but not to that of *R. prowazekii*. These results confirmed the diagnosis of murine typhus.

This is the first serodiagnosis of murine typhus in Vietnam since the 1960s (*1*–*5*). Since rats inhabit the area where the patient acquired the illness, murine typhus seems to have

Table. IFA titers of the patient sera and the cross-absorption test*

Day of illness	Immunoglobulin class	Antigen for absorption	Antigen for IFA titration			
			TG rickettsiae		SFG rickettsiae	
			R. typhi†	*R. prowazekii*‡	*R. japonica*§	*R. conorii*¶
10	IgG	(–)	320	320	<20	20
	IgM	(–)	160	40	20	20
14	IgG	(–)	1,280	640	<20	40
		R. typhi	<20	<20	<20	<20
		R. prowazekii	160	0	<20	<20
	IgM	(–)	640	320	80	80
		R. typhi	<20	<20	<20	<20
		R. prowazekii	160	<20	<20	<20
24	IgG	(–)	640	640	<20	40
	IgM	(–)	640	320	80	80

*IFA, indirect immunofluorescence assay; IgG, immunoglobulin G; TG, typhus group; SFG, spotted fever group.
†Strain Wilmington.
‡Strain Breinl.
§Strain YH.
¶Strain Malish 7.

occurred sporadically or endemically but to have been undiagnosed since the 1960s, maybe because it was thought to have been eradicated and thus widely forgotten. This case was the first imported into Japan since the 1940s, when many Japanese soldiers and residents who returned from abroad had the disease.

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Epidemic Risk after Disasters

To the Editor: We conduct communicable disease risk assessments after humanitarian emergencies, including natural disasters, and would like to clarify the findings of Floret et al. (*1*) regarding the risk for epidemics in certain disaster settings. Natural disasters that do not result in population displacement, regardless of type of disaster, are rarely associated with increased risk for epidemics. However, large-scale population displacement, with consequent overcrowding in temporary settlements and disruption of water supply and sanitation, are indeed associated with increased risks for communicable disease transmission. This distinction is well documented (*2–4*). Increased communicable disease incidence after flooding and cyclones has been particularly well described (*5*,*6*). In addition, after a disaster of any type, epidemics may go undetected because of poor surveillance or because baseline surveillance data for diseases (such as dengue fever or malaria) are unavailable.

Although we agree with the authors that media reports are often exaggerated and that the risk for epidemics after certain types of natural disasters (e.g., volcanic eruption) is low, we believe the findings are somewhat misleading. Postdisaster communicable disease incidence is related more closely to the characteristics of the displaced population (size, health status, living conditions) than to the precipitating event.

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In response: Watson et al. stressed some points that may be important determinants in assessing the risk for epidemics following natural disasters (*1*). We agree that large-scale population displacement, with overcrowding and water disruption, is clearly a risk factor for disease transmission. This factor was probably the main cause of the measles and diarrhea outbreaks that occurred in the temporary settlements created after the eruption of Mount Pinatubo in the Philippines, as mentioned in our previous article (*2*). However, by studying >600 geophysical disasters (earthquakes, volcano eruptions, and tsunamis) that occurred in the last 20 years, we found that deleterious conditions such as large-scale population displacement with overcrowding and water disruption were uncommon and that epidemics were the exception, not the rule. We agree that some epidemics, especially if they are limited and develop well after the disaster, may remain undetected, as was discussed in our paper (*1*).

However, we do not concur with the opinion expressed by Watson et al. that the incidence of postdisaster infectious diseases is more related to the characteristics of the displaced population than to the precipitating event. Our findings are just the opposite. In contrast to the situation seen with flooding and cyclones, which are sometimes followed by outbreaks of waterborne diseases, such as cholera or leptospirosis, and vectorborne diseases (*3*–*6*), the study we carried out on geophysical disasters did not detect any notable outbreak except for the above-mentioned measles outbreak. Watson et al. illustrated their statement by referring to outbreaks following floods and hurricanes, and not earthquakes, tsunamis, or volcano eruptions. Further work must be carried out on epidemics after floods provoked by heavy rains and hurricanes.

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Community-associated Methicillin-resistant *Staphylococcus aureus*

To the Editor: Community-associated (CA) methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is a global emerging threat (*1*–*7*). Accurate measures of the extent of CA-MRSA are critical to allocate resources, guide control measures, and inform prescribing practices (*8*). We assessed the utility of administrative databases, a computerized clinical data repository, and an electronic rule to enhance surveillance for CA-MRSA at Stroger (Cook County) Hospital, a 464-bed public safety net hospital in Chicago, and its associated clinics—all part of the Cook County Bureau of Health Services (CCBHS).

Using data collected within the Chicago Antimicrobial Resistance Project computerized clinical data repository (*9*) from September 1, 2001, to August 31, 2004, we developed an electronic rule to define persons with CA infection with *S. aureus*. This rule used the electronic records of all persons from whom MRSA or methicillin-susceptible *S. aureus* (MSSA) had been identified in cultures of soft tissue, pus, bone, or joints. Infections from patients who met the following electronic case definition were designated CA: 1) culture obtained as an outpatient or within the first 3 days of hospitalization, 2) no clinical culture with MRSA in the last 6 months, 3) no hospitalization or surgeries within 1 year, and 4) no hemodialysis. All other infections were defined as healthcare associated. Data for microbiology results, demographics, and recent surgery or hospitalization were linked by a unique patient identification number. Dialysis use was detected by the use of biochemical tests obtained around the time of dialysis or of hemodialyis-related ICD-9 procedure codes (39.27, 90945, 39.95, 90935, 54.98, 39.43, 39.42, or 38.95). Because the electronic data sources were complete for the period specified, absence of data for a patient was considered to be due to the absence of exposure, not missing data.

Using the electronic case definition and data repository, we randomly selected 100 patients with putative CA- and 100 with putative healthcare-associated *S. aureus* infections. The paper charts for these 200 patients were reviewed to validate the designa-

tions of CA- or healthcare-associated infection, by using the same criteria as for the electronic rule. To ensure blinding for manual chart reviews, all references to results of the electronic rule were removed from data collection instruments. Using information obtained from chart review as the standard, we determined sensitivity and specificity of the electronic rule and calculated agreement (κ statistic) between manual and electronic reviews. To ascertain data sources of most value in detecting healthcare exposures, we examined data tables required for each type of exposure and for coincident exposures to develop more parsimonious data requirements.

During the study period, 714 (386 MSSA and 328 MRSA) healthcare-associated and 1,222 (518 MRSA and 704 MSSA) CA infections occurred; all electronic data elements were available for all patient encounters that occurred within CCBHS. Sampling yielded 47 CA- and 52 healthcare-associated MRSA infections and 53 CA- and 48 healthcare-associated MSSA infections.

The electronic case definition performed well when compared with chart review. All 100 healthcare-associated infections identified electronically were confirmed by manual chart review as classified correctly. Among the 100 community-associated infections identified electronically, 3 (3%) were determined by chart review to have been misclassified: 2 patients had been hospitalized, and a third had surgery within the previous year, all outside CCBHS. The sensitivity of the electronic case definition for community association was 100%; specificity was 97%. The κ statistic was 0.97 (confidence interval [CI] 0.83–1.00), which indicated superior agreement between chart review and electronic rule. For misclassified cases, 1 infection was due to MRSA, and 2 were due to MSSA. The performance characteristics of the rule for CA-MRSA were sensitivity 100%, specificity 98.1%, and $\kappa = 0.98$ (CI 0.78–1.00).

The Table describes data elements required to detect healthcare exposures. The most data-intensive exposure to detect was hemodialysis, which required a search of laboratory and discharge diagnosis databases. Isolates of MRSA were designated healthcare-associated most commonly because of prior hospitalization (523 [73%] of 714) and date of culture (i.e., >3 days after hospital admission) (259 [36%] of 714). With the use of only admission/discharge and microbiology data, 28 patients (90%) who had undergone dialysis and 23 (85%) who had undergone surgery were identified. The use of only admission/discharge and microbiology data would have detected 707 patients, 99% of those who would have been detected by the full algorithm.

Our study had limitations. Chart review may have undercounted healthcare-associated factors and is dependent on clinician histories and documentation. However, retrospective review of paper charts is the principal method that infection control practitioners use to gather information. Also, this study was conducted at a single center that served a population that may have had difficulty seeking care elsewhere. For single hospitals or systems with a less captive population, electronic measures may not function as well until disparate systems can be integrated, i.e., at the level of health departments or through data sharing among regional health information organizations.

In conclusion, using easily accessible data from a computerized clinical data repository, we readily classified *S. aureus* and MRSA infections as CA or healthcare associated. Comparison of the electronic method with manual paper chart review demonstrated high agreement for MRSA ($\kappa = 0.98$). Additional review suggested that use of only 1 or 2 data sources efficiently detected prior healthcare exposures. A major dividend of increased use of information technology in healthcare is application of electronically stored data to improve public health surveillance.

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Table. Data sources for healthcare exposures

Healthcare exposure	Databases needed	Comments
Isolate obtained >3 d after admission	Microbiology laboratory	Microbiology table must contain patient registration date to compare with culture date
Hemodialysis	Discharge diagnoses, biochemistry laboratory	Biochemistry laboratory table must contain location of test to identify dialysis clinic
Prior hospitalization (within 1 y)	Admission, discharge, and transfer data	Query must be able to compare individual patients across admissions
Prior surgeries (within 1 y)	Operating room schedules	May not be readily available in many institutions
Prior isolation of MRSA* (within 6 mo)	Microbiology laboratory	Query must be able to compare individual patients across admissions

*MRSA, methicillin-resistant *Staphylococcus aureus*.

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Cryptosporidium felis Infection, Spain

To the Editor: Coccidian protozoans that belong to the genus *Cryptosporidium* frequently cause gastrointestinal infection in humans and animals and are distributed worldwide. *Cryptosporidium hominis* and the cattle genotype of *C. parvum* are responsible for most human infections. However, other species and genotypes of *Cryptosporidium*, such as *C. felis*, *C. muris*, *C. meleagridis*, *C. canis*, *C. parvum* pig genotype, and *C. parvum* cervine genotype, have also been detected in stool samples of immunosuppressed and immunocompetent patients (*1*). Since 1999, when Pieniazek et al. described 3 cases of *C. felis* infection in HIV-positive patients (*2*), several studies have confirmed that this species can infect humans. Recently, Muthsusamy et al. described *C. felis* infections in 5 HIV-positive persons in southern India (*3*). In this article, we describe our experience with an imported case of *C. felis* infection in Spain.

A pediatrician requested a parasitologic study for an immunocompetent, 4-year-old boy with diarrhea. The child came from an orphanage in Calcutta, India; he had arrived in Spain 10 days earlier after having been adopted by a Spanish family. Stool specimens were tested for a wide panel of enteric pathogens, including bacteria, viruses, and parasites. *Cryptosporidium* oocysts were detected by direct microscopic visualization of the samples, which had been concentrated by formalin–ethyl acetate sedimentation and stained with a modified Ziehl-Neelsen stain. Results were also positive for *Cryptosporidium* for samples tested by using an immunochromatographic (Crypto-Strip, Coris Bioconcept, Gembloux, Belgium) (*4*) and an immunofluorescent assay (Merifluor *Cryptosporidium/Giardia*, Meridian Diagnostics, Cincinnati, OH, USA).

DNA was extracted as described elsewhere (*5*), purified with polyvinyl-pyrrolidone, and stored at –20°C in Tris-EDTA buffer. After DNA extraction, PCR–restriction fragment length polymorphism (RFLP) analysis was performed by using previously described protocols based on the small subunit (SSU) rRNA gene (*6*), with digestion of the amplicon by the restriction enzymes *Ssp*I for species diagnosis or *Vsp*I for *C. parvum* genotype identification. For DNA sequencing, PCR products of the 18S rRNA gene fragments were purified and used for direct sequencing in an ABI377 automated sequencer (Applied Biosystems, Foster City, CA, USA).

RFLP analysis showed a profile distinct from those of *C. hominis* and *C. parvum* cattle genotype and consistent with the published patterns for *Cryptosporidium felis*: 426 and 390 bp with *Ssp*I digestion; 476, 182, and 104 bp with *Vsp*I (*6*). The sequence of the PCR product was determined, and a comparison with all SSU rDNA *Cryptosporidium* sequences available in databanks showed 100% similarity with the homologous fragment of *C. felis* (GenBank accession no. AF112575).

To date, >30 cases of human infection by *C. felis* have been reported in the literature. Only 3 of them have occurred in immunocompetent patients: 2 in the United Kingdom (*7*) and 1 in Peru (*8*). To our knowledge, this is the first case of human *C. felis* infection diagnosed in Spain. The child had been in Spain for only 10 days, no pet animals lived in his new home, and he had not gone to kindergarten. Consequently, the infection was likely acquired in India.

The transmission route for the unusual *Cryptosporidium* species is unclear. In the study by Matos et al., only 1 of 4 immunocompromised patients with *C. felis* had been in close

contact with cats at home (*9*). Unusual cryptosporidial infections are not restricted to immunocompromised hosts, and further investigation of the pathogenicity and epidemiology of these infections is necessary to establish their effect on public health and to identify risk factors for exposure and measures for prevention. The identification of species other than *C. hominis* and *C. parvum* that infect humans, and the transmission routes of such agents, has relevance for better understanding of the epidemiologic features of cryptosporidiosis.

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Correction: Vol. 10, No. 5

In "Syndromic Surveillance in Public Health Practice, New York City," by Richard Heffernan et al., errors occurred. On page 861, in Table 2, the numbers of visits indicated in the headings for columns 3, 4, and 5 are incorrect. In the corrected table, column 3, % age 13–39 y, n = 946,478; column 4, % age 40–64 y, n = 604,707; and column 5, % age ≥65, n = 259,615. Additionally, a footnote has been added to the column 2 heading: *Total number includes 7,266 visits for which patients' ages were unavailable.

The corrected table appears in the updated article at http://www.cdc.gov/ncidod/EID/vol10no05/03-0646.htm#table2

We regret any confusion these errors may have caused.

Corrections: Vol. 11, No. 6

In "Methicillin-resistant *Staphylococcus aureus* Hospitalizations," by Matthew J. Kuehnert et al., an error occurred. In Table 3, columns 3 and 5, the rates shown for hospitalization with *S. aureus* and MRSA-related discharge diagnoses were per 10,000 discharges, rather than per 1,000 discharges, as indicated.

The corrected table appears in the updated article at http://www.cdc.gov/ncidod/EID/vol11no06/04-0831.htm#table13

We regret any confusion this error may have caused.

The Access Principle: The Case for Open Access to Research and Scholarship

John Willinsky

MIT Press, Cambridge, Massachusetts, 2005
ISBN: 0262232421
Pages: 287; Price: US $34.95

Emerging Infectious Diseases helped pioneer open-access publishing by launching free online and print editions simultaneously in 1995. A decade later, perhaps half of the 50,000 scholarly journals are available online; however, access to the contents generally requires a subscription. Although many journals are experimenting with enhanced-access models, such as offering open access to a small selection of articles (e.g., Lancet) or making archived articles freely available 6–12 months after publication (e.g., New England Journal of Medicine), only ≈20% of all research articles are open access, and many of these are available only as self-archived manuscripts on authors' personal websites. Meanwhile, journal subscription rates continue to escalate, strapping library budgets and restricting circulation.

In this book, John Willinsky, professor of literacy and technology at the University of British Columbia, argues that access to the results of research and scholarship are a public good: information shared is not diminished; in fact, only when shared does it become knowledge. The access principle states that a commitment to research entails a responsibility to circulate the results as widely as possible.

Each chapter in the book presents this principle from a different perspective, making a case for open access on philosophical, ethical, practical, economic, and technical grounds. In each instance, the contentions of open-access critics are carefully dissected, exposed, and refuted with timely and relevant data. Individual researchers concerned about losing prestige and officers of professional societies concerned about losing subscription revenue might find these arguments particularly interesting. For example, the evidence from physics, a field with well-established open-access publishing conventions, suggests that open-access articles are cited more often (i.e., higher impact factor) than those available only to subscribers. Analyses of scholarly association budgets and journal management economics, which appear among the useful appendices at the end of the book, suggest that alternative publishing models could be more cost-effective than the status quo.

The author does not overlook the irony of publishing this work as a book that costs $34.95, but I tend to agree that the book is still the best medium for a "thoroughgoing treatment of an issue in a single sustained piece of writing." This book is for scholars and professionals who are interested in the idea of open access but are not yet convinced. Those who read it are likely to be surprised, engrossed, informed, and perhaps persuaded.

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etymologia

Trypanosoma

[tri-pan′′o-so′mə]

From the Greek *trypanon*, "borer," plus *soma*, "body," *Trypanosoma* is a genus of hemoflagellate protozoa, several species of which are pathogenic in humans. *Trypanosoma cruzi*, the etiologic agent of Chagas disease, is transmitted from its vector to humans in the insect's feces, not its saliva, as is the case with most other arthropodborne organisms, including *Trypanosoma brucei*, the etiologic agent of sleeping sickness.

Sources: Dorland's illustrated medical dictionary. 30th ed. Philadelphia: Saunders; 2003 and wikipedia.org

Daudi E.S. Tingatinga. Leopard (2006). Acrylic bicycle paint on canvas (73.66 cm × 73.66 cm).
Courtesy of U*Space Gallery (www.uspacegallery.com), Atlanta, Georgia, USA

"He Who Dines with the Leopard Is Liable To Be Eaten"

– Proverb of the Fipa, southwest Tanzania

Polyxeni Potter*

Bicycle paint—thick, slow to dry, and available—proved just the right medium for working on masonite, canvas of choice for tingatinga art. The concept originated with Edward Saidi Tingatinga (1932–1972), a Mozambican who lived and worked in Tanzania. Tingatinga had scant opportunity for artistic training or academic pursuits. Growing up in a farm family, he was preoccupied with survival, doing odd jobs to escape poverty. Talented nonetheless and energetic and living in East Africa, a region rich in artistic traditions (music, poetry, dance, sculpture), he drifted toward creative work: music at first, then embroidery, weaving, and house paintings—a type of continuous decoration wrapped around dwellings, forcing the viewer to circle the work to experience it fully (*1*). In the 1960s, he started painting animals and other single motifs in a consistent and colorful but spare style.

Tingatinga's preference for bicycle paint involved more than availability. Because it dried slowly, the paint required separate processing of the background before other elements could be superimposed, creating sharp color contrasts and adding to the abstract quality of the work. The characteristic consistency also facilitated shading and color variation (*2*).

The paintings were taken to Oyster Bay, an affluent section of Dar es Salaam, Tanzania's commercial capital, and sold well at the Morogoro stores, which catered to visitors. The boldness and excitement of the work attracted attention. Tingatinga was invited to join the National Arts Company and was able to devote all his time to painting. He soon employed several apprentices, who eventually produced independent work in the same style (*3*). He died young, but his work, continued by his relatives and students, burgeoned into a local genre, widely imitated and identified with the region. The art became known beyond Tanzania, in Kenya and other parts of Africa, as well as Norway, Sweden, Finland, Denmark, and other parts of Europe. It was put on display in Tanzania's national museum, the first domestic exhibition to be included and the first by a self-taught artist.

Tingatinga painted a theme prominent in southern and eastern African art, "the big five" large animals (elephant, lion, rhinoceros, buffalo, leopard) common in this region. "Naïve" but intuitively in touch with the elements of this

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and other African themes, the artist chose vibrant color and abstraction over naturalistic description, representing rather than depicting his subjects. This element of abstraction in African art (often seen in traditional masks or wood, ivory, stone, and other carvings), as well as reliance on bold dramatic color, was extremely influential in the development of modern art, inspiring such masters as Pablo Picasso, Henri Matisse, and Amadeo Modigliani (*4*).

Tingatinga's work contained elements of the Makonde and Swahili cultures and coastal East African design. A celebration of nature and ordinary daily activities, it was characterized by borderless motifs and direct presentation. Seemingly uncomplicated, it was symbolic and metaphorical, using content, color, and design to represent beliefs and ideas. Now as ubiquitous as visitors to Africa, tingatinga art is widely reproduced on anything from small ceramic tiles to large masonite squares and is part of the global "tourist" or "airport trade," prompting some to question its authenticity.

The leopard, on this issue's cover, was painted by Daudi, Tingatinga's son, who has been recreating some of his father's most representative works (*5*). The ferocious, carnivorous mammal it represents is stealthy and shrewd, solitary, and loyal only to its cubs. Eschewing a tawny coat, this one wears black and sports the circular rosettes of East African leopards. Awash in the primary colors of the region's Bantu societies: red, white, and black—triad of the spirit world (e.g., black for African; white for Arab, European; red for spirits of the dead) (*6*)—it dominates the painting from edge to edge, soft on its toes, ready to leap.

The zoologic profile—appearance as well as behavior of this wild cat—illuminates its symbolism, so prevalent in African cosmology and lore. Much has been made of the leopard's maternal bias and high, yet secondary (to the lion), position in the animal kingdom. Its speckled black-and-white pelt has stood for contrast: day and night, wet and dry, human and wild. Its unpredictable temperament and trickery are symbols of, among other topical scourges, despotic rule. Symbolism extends to leopard skin garments and the power of those who wear them, whether heroic figures, political leaders, or spiritual healers (*7*).

Symbolic elaboration of the leopard, old as enigmatic spots on prehistoric caves and diverse as variegated fur, does not fail to apply to emerging diseases. And the subtle nuances of Tingatinga's intuitive expression capture more than Africa's artistic elements. The expansion of human communities into the wild, a factor in disease emergence, is rampant on all continents, including Africa. The proximity of human and animal habitation, the constant interaction—amicable, hostile, parasitic—and human fascination with the wild place us at the proverbial table with the leopard to partake at our own risk.

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EMERGING INFECTIOUS DISEASES

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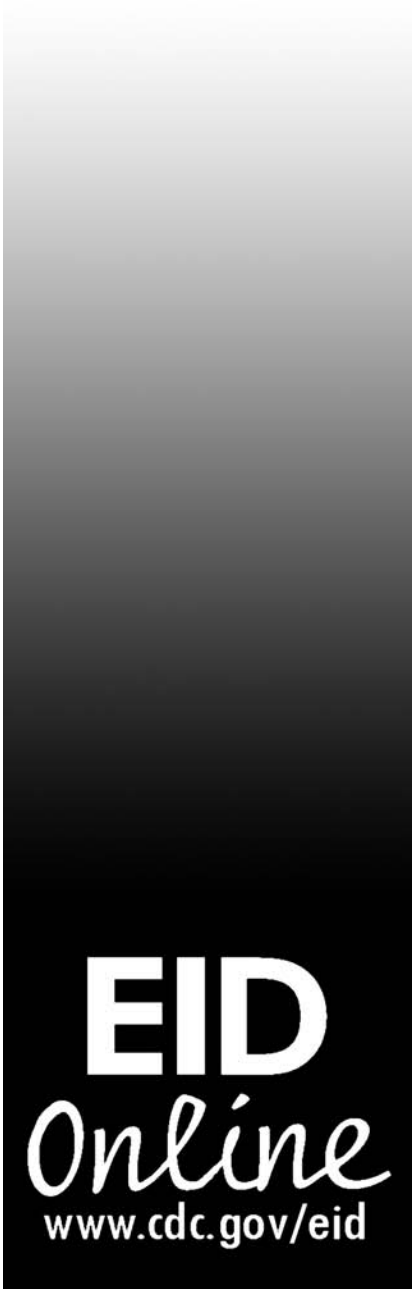
Complete list of articles in the October issue at http://www.cdc.gov/ncidod/eid/upcoming.htm



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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. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (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 of the conclusions, 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), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only 2. 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 2. Report laboratory and epidemiologic results within a public health perspective. 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 of the conclusions, 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 no more than 1,200 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 2); tables (not to exceed 2); and a brief biographical sketch of first author—both authors if only 2. 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 no 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. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

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. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.