EMERGING INFECTIOUS DISEASES®

March 2011

Tuberculosis

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On the Cover

Edvard Munch (1863–1944)
The Sick Child (1907)
Oil on canvas
Dimensions: support (1,187 mm × 1,210 mm)

The Munch Museum/The Munch-Ellingsen Group/ Artist Rights Society, NY. Image copyright, Tate, London, 2011

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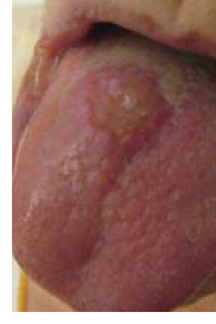

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Bridging Implementation, Knowledge, and Ambition Gaps to Eliminate Tuberculosis in the United States and Globally

Kenneth G. Castro and Philip LoBue

We reflect on remarkable accomplishments in global tuberculosis (TB) control and identify persistent obstacles to the successful elimination of TB from the United States and globally. One hundred and twenty nine years after Koch's discovery of the etiologic agent of TB, this health scourge continues to account for 9.4 million cases and 1.7 million deaths annually worldwide. Implementation of the Directly Observed Treatment Short-course strategy from 1995 through 2009 has saved 6 million lives. TB control is increasingly being achieved in countries with high-income economies, yet TB continues to plague persons living in countries with low-income and lower-middle–income economies. To accelerate progress against the global effects of disease caused by TB and achieve its elimination, we must bridge 3 key gaps in implementation, knowledge, and ambition.

As we commemorate World Tuberculosis (TB) Day, March 24, we pause to reflect on remarkable accomplishments in eliminating TB in the United States and other parts of the world and to identify persistent obstacles to its eventual elimination. World TB Day marks the day when, in 1882, Robert Koch delivered his lecture to the Physiologic Society of Berlin announcing the discovery of the tubercle bacillus as the etiologic agent of TB (*1*). At the time, TB was estimated to account for one fifth to one fourth of all deaths in Europe. One hundred twenty-nine years later, TB is increasingly under control in most countries with high-income economies (*2*) yet continues to afflict persons living in countries with low-income and lower-middle–income economies (*3,4*). The World Health Organization (WHO) reported an estimated 9.4 million incident TB cases and 1.7 million deaths in 2009. Existing evidence-based interventions for TB control that have been successfully implemented from 1995 through 2009 have saved 6 million lives and alleviated much human suffering (*3*). Yet, by 2009 only an estimated 63% of annual incident TB cases were being detected and reported; of these, 86% were successfully treated (*3*). To accelerate progress against the global effect of disease caused by TB and to achieve its elimination, we must bridge 3 key gaps in implementation, knowledge, and ambition.

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Implementation Gap

In his 1963 lecture delivered at the Postgraduate Medical School in London, Wallace Fox observed that remarkable progress in the chemotherapy of TB had been achieved over the prior decade "in the technically advanced countries" (*5*). In contrast, he remarked that nonindustrialized countries "have derived very little benefit from the progress." Fox cited 2 reasons for this lack of progress: a shortage of medical resources and "little attempt to adapt present knowledge to their specific problems" (*5*). During Fendall's 1972 presentation at the Symposium on the Teaching of Teaching Tropical Medicine, an epitaph is suggested to describe medicine throughout the 20th century: "Brilliant in its scientific discoveries, superb in its technological breakthroughs, but woefully inept in its application of knowledge to those most in need" (*6*). Fendall further suggested "all that remains is the problem of translating what is current common knowledge and routine medical and health practice to the other two thirds of the world: the 'implementation gap' must be closed."

Globally, this implementation gap has been closing as a result of reliance on the evidence-based strategy for TB control, originally known as Directly Observed Treatment Short-course (DOTS). This strategy was initially based on diagnostic and treatment recommendations derived from randomized controlled trials, conducted largely by the British Medical Research Council (*7*) and the US Public Health Service, which established the efficacy and safety of drugs against TB (*8*). Additionally, the basic elements of the strategy were defined and field tested under mutual assistance programs between host countries and the International Union Against Tuberculosis and Lung Disease (*9*). The DOTS strategy was endorsed by consensus derived in technical advisory bodies and promulgated by WHO and the global Stop TB Partnership. Its widespread implementation has been more recently facilitated by resources from governments; the Global Fund for AIDS, Tuberculosis, and Malaria; and the President's Emergency Plan for AIDS Relief (*10–12*). Furthermore, TB control has been demonstrated to be among the most cost effective of health interventions (*13*).

The original DOTS strategy contained 5 basic elements: 1) secure political commitment with adequate and sustained financing; 2) ensure early case detection and diagnosis through quality-assured bacteriology; 3) provide standardized treatment with supervision and patient support; 4) ensure effective drug supply and management; and 5) monitor and evaluate performance and effects. This strategy has now been expanded to contain additional elements to confront other evolving needs, such as addressing HIV-associated TB, and multidrug-resistant and extensively drug-resistant TB; contributing to strengthening health systems; engaging all providers (public, voluntary, and private) and affected communities; promoting use of the International Standards for TB Care; and enabling and promoting research (*14*,*15*).

The advances achieved with DOTS from 1995 through 2009 include treating nearly 49 million persons and curing 41 million with TB, which was accompanied by a peak in global TB trends in 2004 followed by a relatively slow decline (*3*). These advances notwithstanding, TB continues to hold its dubious place as a leading infectious killer of young adults, and the disease preys on the most vulnerable persons in many parts of the globe (*16*). These populations are known to have difficulty in accessing available diagnostic tests and in obtaining curative short-course therapeutic regimens that require ≥6 months of multiple drugs to achieve the desired outcomes. Even when all countries of the world have adopted policies consistent with the DOTS strategy, a sizable proportion of estimated cases (≈37%) are undetected, and those infected are likely not receiving optimal treatment regimens. Efforts must now focus on tackling social determinants of illness associated with TB by expanding and facilitating access to impoverished persons in densely populated urban areas and remote villages.

In addition to partnering with all health providers (e.g., private, public, voluntary, traditional healers) to facilitate access to care, those concerned with public health must concentrate on subtleties such as optimizing the number of clinics or dispensaries offering diagnostic and therapeutic services, providing patient-convenient hours of operation, recognizing difficulties with distance and transportation, and minimizing out-of-pocket expenditures (including lost wages) for transportation, child care, and diagnostic services. An analysis from India has reported that 72% of TB patients who had a low standard of living (e.g., earning US $1–$2/d) first saw private providers and spent, on average, $145 before starting treatment with the Revised National TB Program, thus documenting the devastating economic toll incurred by poor persons with TB (*17*). Engagement of affected communities will also prove crucial to create educated consumers of services. Public communication campaigns will help educate persons about the signs and symptoms of TB, provide information about where to access quality services and drugs, alleviate stigma, and create the demand for these basic health services from all providers of care and government decision-makers.

Other scientific advances that have lagged behind in implementation include the use of universal genotyping of *Mycobacterium tuberculosis* clinical isolates as a way to understand and interrupt chains of recent and ongoing transmission and the use of universal drug susceptibility testing with liquid culture media that reduce turn-around times by several weeks (available in the United States since 1994) for timely surveillance of drug resistance trends and to guide optimal treatment regimens. Most recently, technologic advances have demonstrated the ability to rely on detection of bacterial DNA by PCR. The WHO policy recommendation to rely on Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) for same-day diagnosis accentuates and magnifies this implementation gap (*18*).

A growing concern has to do with the gap in successfully addressing concurrent conditions associated with TB, such as HIV, diabetes, smoking, indoor air pollution, alcoholism, and malnutrition (*16*). This more holistic approach provides an ideal way to benefit both individual and public health, and secondarily to strengthen health systems. When modeled after the basic principles that underpin TB control, the combined interventions will provide platforms for planning, service delivery, analysis, accountability, and corrective actions.

In the zeal to bridge the implementation gap, we must avoid past false dichotomies. There are those who see the way forward as limited to securing investments and channeling all resources to expand access to available diagnostic services

and curative drugs. Available tools are relatively blunt and limited, especially for effectively addressing HIV-associated TB and multidrug-resistant and extensively drug-resistant TB. In tackling urgent unmet needs, we must honestly acknowledge existing limitations and not ignore the need to bridge the immense knowledge gap in TB. Otherwise, we risk interventions that lack innovation, creativity, and do not keep pace with technological advances that could accelerate the path to elimination.

Knowledge Gap

There remain critical areas of collective ignorance with regard to *M. tuberculosis.* These include knowledge of rapid, simple, and inexpensive methods of detection; molecular mechanisms of resistance to chemotherapy; virulence; host defense correlates of susceptibility to and protection against the organism; and optimal targets for development of new antimicrobial drugs.

Until the past 2 decades, definitive detection of *M. tuberculosis* relied exclusively on culture, which takes weeks because of the requisite generation time of 18–24 hours, giving rise to the apt descriptor of *M. tuberculosis* as "slow growing" bacteria. In low resource settings, even culture may not be available and diagnosis must be based on smear microscopy, which fails to detect nearly half of patients with TB (*14*). Advances in molecular biology and, ultimately, the sequencing of the *M. tuberculosis* genome led to rapid molecular methods of detection that, although reasonably accurate, were cumbersome and expensive (*19*,*20*). Only relatively recently has a promising new molecular diagnostic test become available, the Xpert TB/RIF, that is both simple and accurate (*21*). However, even with concessionary pricing for low-income countries, cost still remains an issue.

Similarly, detection of drug resistance almost solely relies on phenotypic culture-based methods. Here, also, advances in molecular biology are moving the field forward, but the situation is more complex than for detection of the organism. Fortunately, for the most important anti-TB drug, rifampin, >95% of resistance can be attributed to mutations in 1 gene, which has greatly simplified the development of molecular tests to detect rifampin resistance (*22*). Commercial assays that use line-probe and molecular beacon technologies have been produced that are rapid and accurate (*22*,*23*). However, for other first-line drugs (such as isoniazid and ethambutol) and second-line drugs, the molecular mechanisms of drug resistance have only been partially elucidated, inhibiting the development of rapid molecular assays for these drugs (*24*). Thus, there remains a heavy reliance on inefficient and slow culture-based phenotypic methods.

Virulence of *M. tuberculosis*, especially variation among strains, is also poorly understood. There is evidence suggesting some strains may result in higher rates of disease progression, treatment failure, and relapse (*25*). Identifying genetic markers of *M. tuberculosis* virulence would enable additional attention to be focused on patients infected with strains manifesting such markers and who are therefore at the greatest risk for poor outcomes.

Our lack of understanding of host defense correlates of susceptibility to and protection against *M. tuberculosis* has stymied progress in 2 key areas: vaccine development and prevention through treatment of latent TB. A vaccine that uses an attenuated strain of *M. bovis* (*M. bovis* BCG) has been available for nearly a century and is one of the most widely used vaccines in the world. Although the vaccine does offer substantial protection against dissemination of *M. tuberculosis* infection in children, it only provides modest and highly variable protection against TB in general (*26*,*27*). Clearly, more efficacious and safe vaccines are needed; these are only likely to be produced through a better understanding of immunologic mechanisms and correlates of protection. A related knowledge gap is the lack of understanding of why only a small fraction (≈5%–10%) of persons infected with *M. tuberculosis* later exhibit disease (*28*). It is evident that immunocompromised persons (e.g., HIV infected or receiving tumor necrosis–α inhibitors) are at greater risk, but we have little knowledge of why certain persons with apparently healthy immune systems experience progression to illness (*28*). This results in treating 10–20 persons with latent TB for every 1 that will have the infection progress to disease. Given the length of optimal treatment (9 months) and potential toxicity (liver injury), this intervention is obviously suboptimal and could be made much more efficient if it could be targeted to persons at the highest risk of becoming ill. Thus, there is a crucial need to find genetic and immunologic markers that confer increased susceptibility to progression.

Standard TB treatment requires multiple drugs for ≥6 months' duration (*29*). These drugs have multiple and overlapping toxicities. For drug-resistant TB, treatment consists of more toxic, less effective second-line drugs that must be taken for 18–24 months (*29*). Some patients with extensively drug-resistant TB have been described as having run out of realistic therapeutic options and thus resemble TB patients in the pre–antimicrobial drug era. Additionally, persons with latent TB who are not ill tend to have a difficult time completing the 9 months required for isoniazid treatment (previously described as preventive therapy or chemoprophylaxis). Safe and effective regimens that could be administered intermittently and/or within 3 months are under study and show promise (*30*). All these factors underscore the need for new medications that are better tolerated and can produce a cure in less time. Given that drug toxicity and resistance are often class effects, development of new classes of anti-TB drugs is another

essential research need. Such development, in turn, will likely require identification of new and better drug targets.

Although the knowledge and implementation gaps must be bridged, simply rectifying these inadequacies is insufficient for elimination of TB. Societal resolve and ambition are also required to garner the necessary resources for sustained efforts and effective programs, adapted to local epidemiologic realities.

Ambition Gap

The report from the 1959 Arden House Conference on TB made a daring statement, possibly well ahead of its time, indicating that TB control "has progressed to the point where virtual elimination of the disease as a public health problem appears to be within reach" (*31*). However, it was not until 3 decades later that the Centers for Disease Control published a formal consensus plan for the elimination of TB in the United States (*32*). This plan was ambitious, yet initially naive about the full extent of the effects on TB incidence due to HIV infection; multidrug resistance; institutional transmission of *M. tuberculosis*; and the time lag for the development of new technologies for more effective prevention, prompt diagnosis and detection of drug resistance, and superior treatment of TB. These various factors converged to produce the unprecedented resurgence of TB experienced in the United States during 1985–1992 (*33*). The rapid dissemination of multidrug-resistant TB among HIV-infected persons and their caregivers was accompanied by unacceptably high mortality rates and served as a clarion call to elicit concerted efforts and mobilize new resources to implement the 1992 National Action Plan to Combat Multidrug Resistant TB (*34*). The US Federal TB task force coordinated interagency work and successfully worked with health department-based TB programs across the nation to reverse this trend over ensuing years. In 2000, the Institute of Medicine reaffirmed the goal of TB elimination and recommended additional steps required for accelerated progress, including the need to commit to elimination as a national goal and to monitor progress (*35*).

In recent years, the risk of renewed complacency, resource limitations experienced by local health departments, and the direct effects of global TB on US disease rates (nearly 60% of incident TB cases reported in the United States in 2009 occurred in foreign-born persons) challenges advances to TB elimination in the near future. Bold ambition and expectations with sustained actions are a requisite to successfully eliminating TB in the United States and globally. The report of the 1997 Dahlem Workshop on the Eradication of Infectious Diseases recognizes that "[t]he success of any disease eradication initiative depends strongly on the level of societal and political commitment… Elimination and eradication are the ultimate goals of public health, evolving naturally from disease control. The basic question is whether these goals are to be achieved in the present or some future generation" (*36*).

Smallpox is the only infectious disease in humans that has been successfully eradicated, and this was only achieved by a campaign characterized by global solidarity in planning, collaboration, and concerted action. Few other infectious diseases meet the conditions that favor elimination or eradication (*36*). For the first time in history, the international community has developed an impressive plan to eliminate global TB (*14*). We must seize this opportunity to make added and continued progress against this global health scourge. A nonconformist stance must prevail until TB is eliminated. This frame of mind was aptly recognized in 1963 by William Brown, who advocated for syphilis eradication during the 1960s. He argued that diseases targeted for eradication (or elimination) should attain a "status of intolerability" by both health authorities and the public, such that any occurrence of the disease, "no matter how small," gives cause for immediate action (*37*). Public clamor would help ensure sustained political commitment and ongoing work. With relatively few exceptions, this sense of bold ambition has not characterized those working in TB prevention and control programs. A sense of impoverished will tends to afflict those who work in resource-limited settings. And, to add insult to injury, there is a natural human propensity toward complacency when progress is being made and a disease is perceived to be under control. Attention and resources risk being diverted to address other pressing health needs. A 1962 Time Magazine article on syphilis resurgence and prospects for eradication demonstrates Dr Brown's full grasp of this reality when he stated: "As a program for the control of a disease approaches the end point, meaning eradication, it is not the disease but the program that is the more likely to be eradicated" (*38*).

Clearly, we must boldly aspire to achieve the elimination of TB and commit to making it a reality in the United States and throughout the globe. Bridging all 3 gaps in implementation, knowledge, and ambition should become mutually reinforcing to achieve the desired results.

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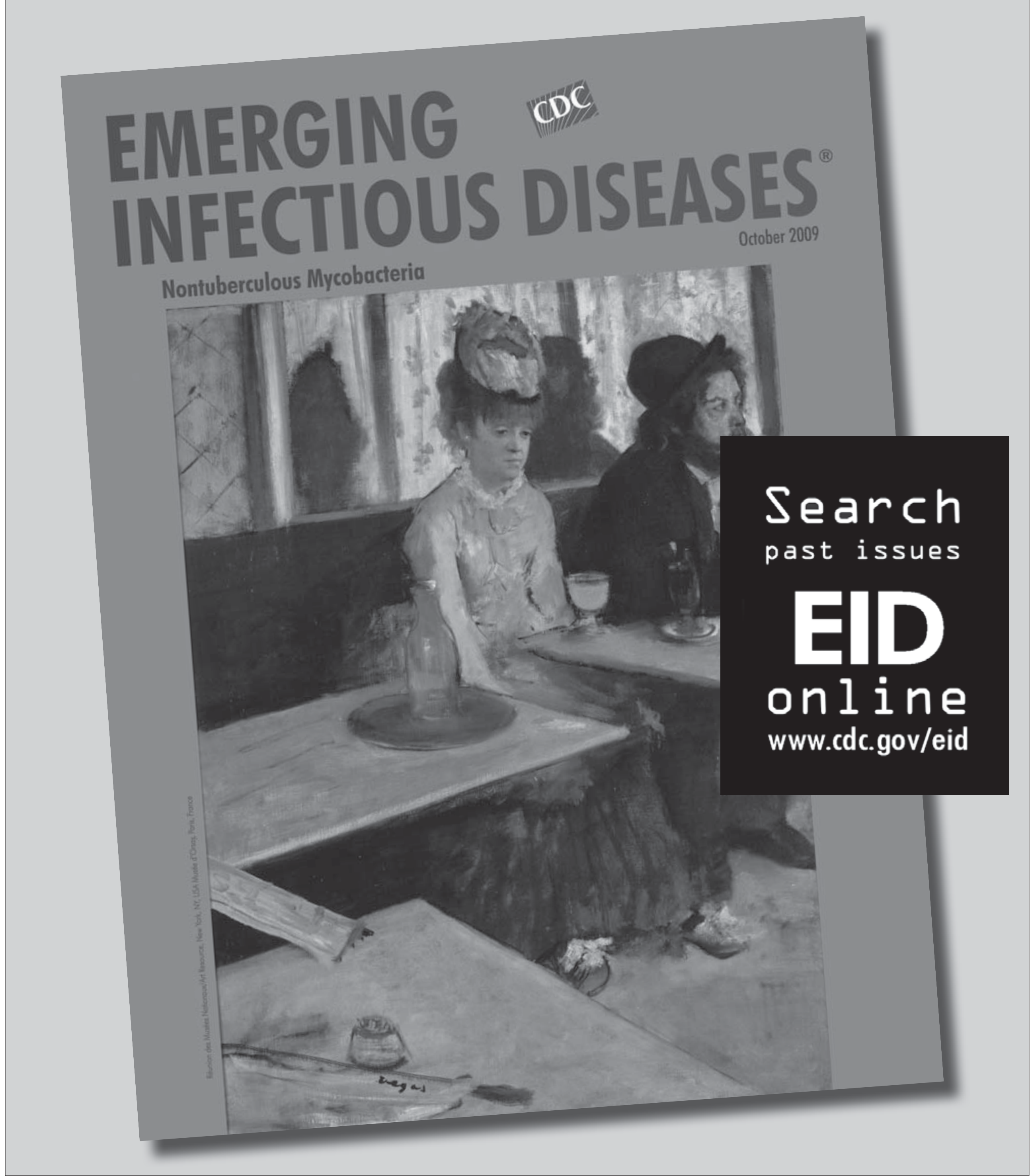

Nontuberculous Mycobacteria in Respiratory Tract Infections, Eastern Asia

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To characterize the distribution of nontuberculous mycobacteria (NTM) species isolated from pulmonary samples from persons in Asia and their association with pulmonary infections, we reviewed the literature. *Mycobacterium avium* complex bacteria were most frequently isolated (13%–81%) and were the most common cause of pulmonary NTM disease (43%–81%). Also pathogenic were rapidly growing mycobacteria (*M. chelonae, M. fortuitum, M. abscessus*). Among all NTM isolated from pulmonary samples, 31% (582/1,744) were considered clinically relevant according to American Thoracic Society diagnostic criteria. Most patients were male (79%) and had a history of tuberculosis (37%). In Asia, high prevalence of rapidly growing mycobacteria and a history of tuberculosis are distinct characteristics of pulmonary NTM disease. This geographic variation is not well reflected in the American Thoracic Society criteria for NTM infections and could be incorporated in future guidelines.

Nontuberculous mycobacteria (NTM) are common in the environment and have been isolated worldwide (*1*). They are increasingly recognized as pathogens in humans. Pulmonary disease is the most common manifestation (*2*) and is thought to result from aerosol inhalation. Because of their omnipresence in the environment, isolation of NTM from the respiratory tract does not, per se, indicate NTM disease. Therefore, the American Thoracic Society (ATS) has established diagnostic criteria to help distinguish between contamination and true NTM disease (*1*).

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Although NTM are present worldwide, much of the literature on pulmonary NTM disease comes from industrialized countries, mainly Europe, North America, and Japan. Differences in the NTM species distribution in clinical samples and disease have been noted among these regions (*3*); by extrapolation, these characteristics in other parts of the world probably differ as well.

Asia has a long history of NTM research, both clinical and fundamental. Tsukamura et al. have reported on pulmonary NTM infections in Japan dating back to the early 1970s (*4*). Yet because many studies were not reported in English, knowledge of the distribution of NTM species in Asia is limited. However, these data will enhance our understanding of NTM diversity between and within species and their association with NTM disease in humans. For this reason, we searched the literature on clinical NTM isolation and disease from different regions in Asia and compared our findings with previously published data from other regions.

Literature Search

From March 2009 to December 2009, we searched PubMed (www.ncbi.nlm.nih.gov/pubmed) for English-language articles about nontuberculous mycobacteria in Asia. The search strategy was as follows: "mycobacteria, atypical" [MeSH] AND "Asia" [MeSH] OR "atypical mycobacterium infections" [MeSH] AND "Asia" [MeSH]. We chose the term Asia to incorporate the following countries: Brunei, Cambodia, East Timor, Indonesia, Lao People's Democratic Republic, Malaysia, Myanmar, Philippines, Singapore, Thailand, Vietnam, Bangladesh, Bhutan, India, China, Hong Kong, Japan, South Korea, Mongolia, and Taiwan.

We found 256 citations. If we considered the abstract to be relevant, we obtained a full copy of the article; we contacted authors if full-text articles could not be retrieved. Furthermore, the reference sections were screened for other eligible citations. We considered 67 articles to be relevant, of which 37 were excluded for the following reasons: 11 were case reports, 7 referred to disseminated NTM infections, 6 were not in English (5 in Japanese, 1 in Chinese), 6 did not concern pulmonary NTM, 4 were reviews, and 3 represented neither epidemiologic data nor clinical cases. From the remaining 30 articles (*4–33*), the following data were abstracted for this review: country, research setting, NTM species, clinical features of the patients, and radiographic data. All articles were screened to determine whether ATS diagnostic criteria for the determination of clinical relevance of NTM isolations applied (*1*). Cases consistent with the ATS diagnostic criteria were considered clinically relevant.

Search Results

We identified 30 English-language articles about the epidemiology and clinical relevance of NTM isolates in Asia (*4–33*). We found data from China (*5*), Hong Kong (*6*), India (*7–13*), Japan (*4*,*14–20*), South Korea (*21–24*), Singapore (*25*), Taiwan (*26–29*), and Thailand (*30–33*). Most articles used a combination of methods to identify NTM species. Biochemical and phenotypic analysis (n = 11) were used most frequently, followed by molecular tools (n = 4) or a combination of both (n = 5). From 10 articles, the exact methods of species identification could not be determined. The use of identification methods differed over time; biochemical and phenotypic analyses were mostly used during 1966–1990 (6 of 7 studies), whereas use of molecular tools increased during 1990–2009 (Table 1). The number of different species isolated did not differ between the 2 periods. However, most species identification by biochemical testing depended heavily on 1 study from India (*11*).

Epidemiology

Regardless of clinical relevance, 25 articles reported on NTM isolates from pulmonary samples (Figure 1) (*4–13*,*17–22*,*25–33*). In general, *Mycobacterium avium* complex (MAC) was most frequently (67%) isolated, although it predominated in northeastern Asia (South Korea and Japan). Exact species identification of MAC (now *M. avium*, *M. intracellulare*, *M. chimaera*, *M. colombiense*, *M. vulneris*, *M. marseillense*, *M. bouchedurhonense*, and *M. timonense*) (*34*,*35*) was not performed, thereby hampering a more detailed analysis.

Rapidly growing mycobacteria ([RGM] *M. fortuitum* complex, *M. abscessus, M. chelonae*) were frequently identified in pulmonary samples from Taiwan, China, and Singapore (*5*,*25–29*). The overall isolation of RGM in this part of Asia was 16%, making RGM the second most frequently isolated species in this region. Other species frequently encountered were *M. kansasii* (4%) and *M. gordonae* (3.5%). *M. malmoense*, a species regularly encountered in northwestern Europe, was found only 9 times in a single study of 1,000 NTM isolates from India (*11*). Also, *M. xenopi*, which is frequently encountered in Canada and England (*3*), was isolated only 10 times (of 11,987 isolates) and mainly during studies from India (*7*,*11*,*13*,*21*,*27*).

Clinical Relevance

The clinical relevance of pulmonary NTM isolates (Table 2) was mentioned in 10 articles covering a total of 1,744 patients (*6*,*16*,*21*,*23*,*25*,*28–30*,*32*,*33*). No articles covered the clinical relevance of NTM in China. Different criteria were used to define clinical relevance, but all criteria comprised a combination of clinical, bacteriologic, and radiographic criteria. Only 4 studies reported a priori use of ATS criteria (*1*). According to ATS criteria, for 31%

Table 1. Nontuberculous mycobacteria isolated in Asia, 1971–2007*

Species	Before 1990 (n = 1,205)†	After 1990 (n = 7,614)†
Mycobacterium abscessus	–	X
M. aichiense	X	–
M. asiaticum	X	–
M. avium complex	X	X
M. celatum	–	X
M. chelonae	X	X
M. flavescens	X	X
M. fortuitum	X	X
M. gastri	X	X
M. gordonae	X	X
M hemophilum	X	–
M. kansasii	X	X
M. malmoense	X	–
M. marinum	X	X
M. neoarum	X	–
M. parafortuitum	X	–
M. phlei	X	X
M. scrofulaceum	X	X
M. simiae	X	X
M. smegmatis	X	X
M. szulgai	X	X
M. terrae	X	X
M. tokaiense	X	–
M. triviale	X	X
M. thermophilum	X	–
M. thermoresistibile	X	–
M. ulcerans	X	–
M. vaccae	X	X
M. xenopi	X	X

*Data derived from references *4–33*. X, isolated; –, not isolated.
†Proportions of identification methods used (biochemical:molecular) 6:1 before 1990, 5:8 after 1990.

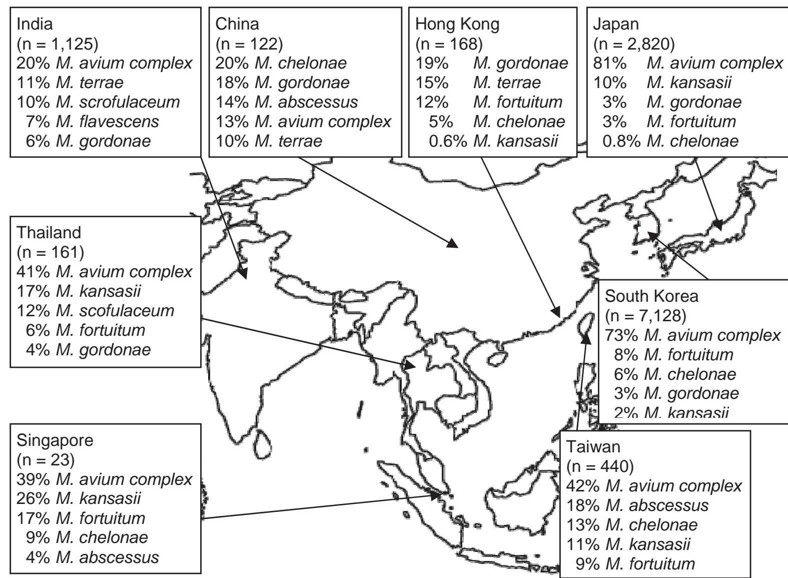

Figure 1. Five most prevalent nontuberculous mycobacteria species found in respiratory specimens, regardless of clinical relevance, Asia, 1971–2007. Data from (*4–13,17–22,25–33*).

(582/1,744) of patients, pulmonary NTM isolates were considered clinically relevant. Relevance varied widely across regions and studies. For example, a study of patients in an intensive care unit in Taiwan found that only 9% of NTM isolates were clinically relevant (*28*); however, in a study of HIV-infected patients in Thailand, relevance rose to 76% (*30*).

Figure 2 shows the distribution of mycobacterial species among patients with definite pulmonary NTM disease in Asia, according to ATS criteria. MAC was the most frequently reported (68% of all cases) cause of NTM disease; RGM were second (14% of all cases). Prevalence of RGM pulmonary infections ranged from 2.6% (Japan) to 44% (South Korea). *M. malmoense* and *M. xenopi* were not reported as causative species in any of these studies in Asia.

We found some discrepancies between isolation frequency (Figure 1) and clinical relevance (Figure 2). For example, in Hong Kong *M. gordonae* was found in 19% of pulmonary isolates regardless of clinical relevance but in only 4% of cases of pulmonary NTM infection. In contrast, in India, higher prevalence of *M. szulgai* was found (1% in all pulmonary samples vs. 7% of all causes of pulmonary NTM). We therefore investigated the clinical relevance of the various NTM species separately, measuring clinical relevance per

Table 2. Clinical relevance of NTM species isolated from pulmonary samples collected in Asia, by country, 1971–2007*

Study area	No. patients	Patients for whom NTM infection was considered clinically relevant, %	Criteria used (year of revision)	Reference
Hong Kong	168	17	ATS criteria (1990)	(*6*)
Japan	357	76†	ATS criteria (1997)	(*16)*
South Korea	794	17	ATS criteria (1997)	(*21)*
South Korea	23	65‡	BTS criteria (1999)	(*23*)
Singapore	23	65	Compatible signs and symptoms, >3 positive sputum specimens, and radiographic features of recent lung disease	(*25*)
Taiwan	111	9	ATS criteria (1990)	(*28*)
Taiwan	169	28§	NTM in >2 samples, new radiographic lesions ,and no other pathogens (definite) or other concomitant bacteria (probable NTM) found	(29)
Thailand	33	76	NTM in sample(s); compatible signs, symptoms, and radiographic features; and no other explanation	(*30*)
Thailand	24	29	Continued NTM isolation, progressive pulmonary disease, and worsening radiographic lesions	(*32*)
Thailand	42	71	Repeated isolation of high numbers of NTM and presence of compatible disease process	(*33*)

*NTM, nontuberculous mycobacteria; ATS, American Thoracic Society (*1*); BTS, British Thoracic Society.
†Only *Mycobacterium avium* complex bacteria were included in this study.
‡Only *M. kansasii* was included.
§14% if only definite NTM were included.

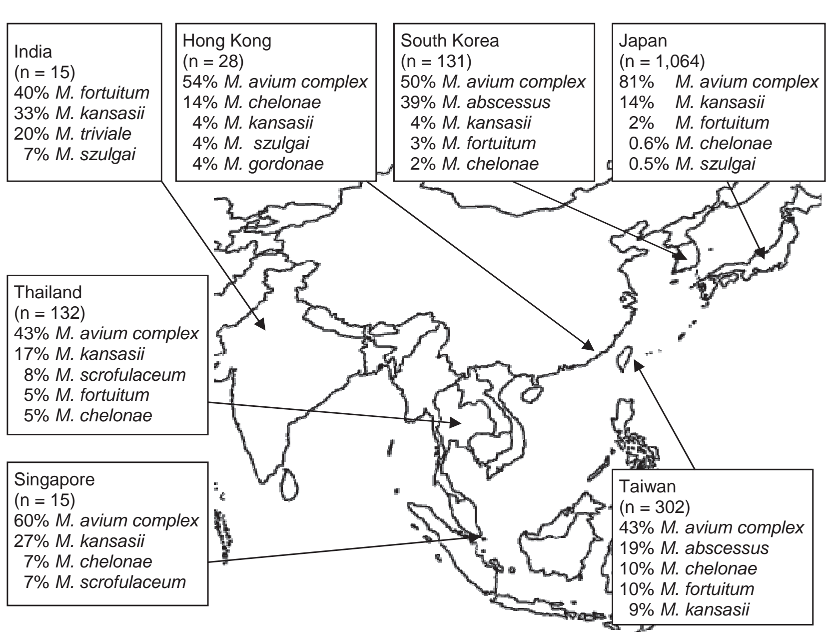

Figure 2. Five most common nontuberculous mycobacteria species causing pulmonary infections, Asia, 1971–2007. Data from (*4,6,10,13,17,19,21,25,27–33*).

species by the percentage of patients meeting ATS criteria (Figure 3). Most clinically relevant species were MAC (56%), followed by *M. abscessus* (35%) and *M. chelonae* (31%). *M. fortuitum*, *M. gordonae*, and *M. terrae* were infrequently reported (1%–3%) as clinically relevant. The varying clinical relevance per species might partially explain the differences between Figure 1 and Figure 2.

Clinical Signs and Radiographic Features

We found information on clinical signs for 689 patients and radiographic data for 1,044 patients (*10,12,14–16,20,21,23–25,27–30,33*). Most patients with pulmonary NTM infections were male (543/689). Because of incomplete data, mean age could not be calculated; 8 studies reported mean ages of 50–70 years. Other characteristics are shown in Table 3. One third of patients had a history of tuberculosis (TB) (252/689). HIV co-infection was less prevalent among patients with localized pulmonary NTM infections (15/280). Clinical signs of NTM disease mimicked those typical of TB: most frequently chronic cough (255/268), followed by hemoptysis (82/268), fever (47/268), and weight loss (40/268). Radiographically, most patients had structural lung disease; 39% (405/1,044) had cavitations and 44% (461/1,044) had bronchiectasis.

Discussion

NTM isolation and disease in Asia have several features. First, a substantial percentage (31%) of patients from whom pulmonary NTM were isolated had clinically relevant NTM disease. This finding is similar to the 33% and 25% found in studies in Canada and the Netherlands, respectively (*2*,*3*). In Asia, NTM may cause substantial pulmonary disease; differences in clinical relevance exist among species (Figure 3), as previously observed (*2*).

Second, MAC was the main cause of pulmonary NTM infection (68% of cases) in Asia. In a key article in 2002, Marras and Daley reviewed the prevalence of pulmonary NTM disease in the world (*3*). They noted a predominance of MAC among the causative agents of pulmonary NTM disease in Asia. Data from Asia were, however, scarce, and their conclusion was mainly based on 1 study (*6*). Our

Table 3. Clinical and radiographic characteristics for patients with pulmonary nontuberculous mycobacteria infections in Asia, 1971–2007*

Characteristic	No. (%) patients
Concurrent conditions, n = 689	
Malignancy, hematologic or solid	79 (11)
Gastrointestinal disease	40 (6)
HIV infection, n = 280 tested	15 (5)
Chronic corticosteroid treatment	18 (3)
Diabetes mellitus	18 (3)
Renal disease	13 (2)
Previous lung disease, n = 689	
Tuberculosis	252 (37)
Chronic obstructive pulmonary disease	62 (9)
Bronchiectasis	47 (7)
Clinical signs, n = 268	
Chronic cough	255 (95)
Hemoptysis	82 (31)
Fever	47 (18)
Weight loss	40 (15)
Radiographic findings, n = 1,044	
Cavitation	405 (39)
Nodular	559 (54)
Bronchiectasis	461 (44)

*Data from (*10,12,14–16,20,21,23–25,27–30,33*).

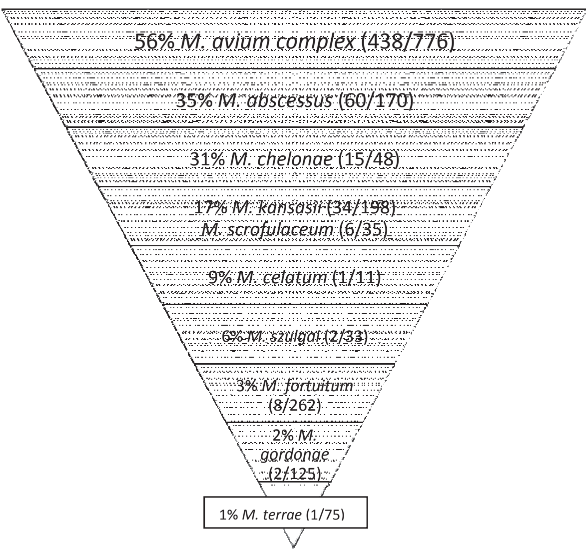

Figure 3. Clinical relevance of pulmonary nontuberculous mycobacterium (NTM) isolates, Asia, 1971–2007. Relevance per species was defined as percentage of patients with pulmonary NTM isolates meeting the American Thoracic Society criteria. Species reported infrequently, i.e., <5×, are not shown. Data from (*6,16,17,21,23,25,29,32,33*).

study supports their conclusion of the predominance of MAC in Asia, which is consistent with its predominance in other parts of the world, namely, North America and most parts of Europe (*3*).

Third, we found that in some regions in Asia, RGM are a major cause of pulmonary NTM disease. This finding contrasts with studies of NTM in other parts of the world (*3*). In a surveillance study from the Netherlands for instance, RGM caused only 3% of all pulmonary NTM infections (*2*). In the United States, this percentage is ≈5% (*36*). In the present review, RGM was found to generally cause 14% of pulmonary NTM infections, but in 3 countries (India, Taiwan, South Korea) this percentage rose to >30% of infections. The fact that RGM were frequently found in pulmonary samples (Figure 1) could reflect higher environmental exposure of RGM in Asia and, hence, higher isolation frequency. The predominance of RGM species may be the result of laboratory practices as well. Ethnic factors may also contribute to susceptibility to different species; i.e., Asian persons could be more susceptible to RGM infection.

Contrary to the high frequency of isolation of RGM species, *M. malmoense* and *M. xenopi*, frequently seen in other parts of the world, were not seen as causative species in any of the studies from Asia. *M. xenopi* has been associated with hot water systems (*1*); as a result, it might be expected to be more rare in Asia, where the water delivery infrastructure is less developed than that in Europe and North America.

A fourth feature of pulmonary NTM disease in Asia—compared with Europe and North America—was the relatively high percentage of patients with a history of TB. This finding might merely reflect the higher incidence of TB in Asia, or it could reflect higher clinician awareness in Asia, such that physicians order *Mycobacterium* spp. cultures in former TB patients with coughing and hence find a relatively higher number of NTM isolates. Alternatively, it could reflect a true predilection of NTM for patients with structural lung disease (*1*) associated with a higher susceptibility to mycobacterial infection in general. The role of TB in the pathogenesis of pulmonary NTM disease is controversial; structural lung damage by a TB infection renders the host vulnerable to NTM disease (*1*), but there are also clues that exposure to TB infers cross-protection to NTM disease (*37*).

Our study has some limitations. The major limitation is the language restriction. The inclusion of languages other than English would probably have increased precision. For instance, during our literature search we came across 5 articles, published as abstracts in PubMed, on NTM infections in Japan. Although certain aspects of these data were already published in the English-language articles we included, we did not have the means to include these non–English-language studies. Nevertheless, our study illustrates the distribution of NTM infections from different geographic areas in Asia and will increase knowledge of the distribution and relevance of NTM species in Asia.

Another limitation is the long time span of the included studies. Because they ranged from 1969 to 2008, they used different decontamination, culture, and identification methods. Data should therefore be considered with caution because of the variety of laboratory procedures used by the several authors to isolate and identify NTM. First, different sample decontamination protocols may determine the yield of NTM by selecting for certain NTM species and inhibiting others (*38*). Second, the introduction of more sensitive liquid media probably increased NTM isolation and perhaps selected for specific species (*26*). Finally, and foremost, the major differences in identification methods used in the studies introduce important biases. Because the taxonomy of NTM has been changing in recent years (*37,39,40*), the different NTM identification methods used in the various studies might have influenced our results; use of molecular tools to identify the historical isolates would probably result in different, more detailed, identifications, especially among the MAC, the *M. simiae* group, and the RGM (*37,39,40*). We did note increased use of molecular tools for NTM identification over time (Table 1), which will, over the next few years, provide us with a more up-to-date overview of NTM species distribution in Asia.

In conclusion, despite the limitations of language and species identification methods, we have described the scale of human pulmonary NTM infections in eastern Asia. MAC bacteria were not only the most prevalent NTM found in pulmonary samples, but they were also the most common cause of pulmonary NTM disease in this geographic region. Distinct epidemiologic and clinical characteristics of pulmonary NTM disease in Asia were found: many patients had a history of TB, and RGM were a frequent cause of pulmonary NTM infections. These distinct characteristics of pulmonary NTM disease in this part of the world are not well reflected in the current ATS criteria on NTM infections and could be incorporated in future guidelines.

Dr Simons is a resident in pulmonary medicine at the Radboud University Nijmegen Medical Centre. His primary research interest focuses on resistance mechanisms in multidrug-resistant tuberculosis.

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Q Fever in France, 1985–2009

Diane Frankel, Hervé Richet, Aurélie Renvoisé, and Didier Raoult

To assess Q fever in France, we analyzed data for 1985–2009 from the French National Reference Center. A total of 179,794 serum samples were analyzed; 3,723 patients (one third female patients) had acute Q fever. Yearly distribution of acute Q fever showed a continuous increase. Periodic variations were observed in monthly distribution during January 2000–December 2009; cases peaked during April–September. Q fever was diagnosed more often in patients in southeastern France, where our laboratory is situated, than in other areas. Reevaluation of the current positive predictive value of serologic analysis for endocarditis was performed. We propose a change in the phase I (virulent bacteria) immunoglobulin G cutoff titer to ≥1,600. Annual incidences of acute Q fever and endocarditis were 2.5/100,000 persons and 0.1/100,000 persons, respectively. Cases and outbreaks of Q fever have increased in France.

Q fever is a worldwide zoonosis caused by the intracellular bacterium *Coxiella burnetii* (*1*). Cases of Q fever in the United States reported during the second Gulf War in Iraq have raised new interest in this disease; Q fever is generally considered a forgotten infectious disease in developing countries (*2*). In a study conducted in France during 1985–1998, the main symptoms of Q fever were classified. This study also reported monthly incidence; peaks in April, May, and June were also identified (*3*). Furthermore, large outbreaks of Q fever in the Netherlands (*4*) and in a school in Israel (*5*) during the past 3 years have brought attention to the frequency of Q fever.

Q fever is primarily transmitted by cattle, goats, and sheep (*6*). Humans are infected by aerosols from parturient fluids of infected livestock. *C. burnetii* is resistant to environmental conditions and can survive for several weeks or months in areas where livestock are present. These bacteria can also be transmitted by wind (*7*).

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The main characteristic of Q fever is its clinical polymorphism. Acute Q fever is defined as primary infection with *C. burnetii*, and ≤60% of infected patients may be asymptomatic. However, Q fever can manifest as an influenza-like syndrome, pneumonia, or hepatitis, and 2% of patients with acute disease are hospitalized. Chronic Q fever is defined as an infection that lasts 6 months and has a phase I (virulent bacteria) immunoglobulin (Ig) titer ≥800. The most common form of chronic Q fever includes endocarditis (73%). Its estimated prevalence among cases of Q fever is 2%–5% (*1*). This pathology often occurs when patients have underlying valvulopathy. Osteomyelitis, vascular infections, and chronic hepatitis have also been described (*2*,*8*). Serologic profiles of patients with acute and chronic Q fever differ. In the acute form, IgM against phase I and phase II (avirulent bacteria) Q fever is observed. In the chronic phase, high levels of IgG against both phases are observed (*9*).

Because symptoms of Q fever are nonspecific (*10*), its diagnosis is still based on serologic analysis and varies with the attention of the attending physician, true prevalence of the disease, and quality of the diagnostic tests (*2*). The definitions of acute and chronic Q fever are based on a previous study conducted when testing for Q fever was performed infrequently (*10*). The French National Reference Center (NRC) database was created in 1985. Because Q fever seemed to be rapidly reemerging in France, we conducted a study that focused on potential causes, seasonality, geographic distribution, and outbreaks of the disease.

Patients

At the NRC, specimens from patients with suspected Q fever are collected routinely. We used these samples for serologic analysis. In cases of endocarditis, heart valves were tested by histological analysis, immunohistochemical analysis, PCR, or cell culture. Serum samples were transported at ambient temperature and valve samples

were transported at –80°C. Since 1985, all serum samples received have been screened by immunofluorescent assay by using a large panel of antigens, including those from *C. burnetii*, *Rickettsia* spp., *Bartonella* spp., and *Francisella tularensis*. When antibody titers indicated infection with *C. burnetii*, monitoring was proposed with a serologic control 3 weeks after the first sample. However, for this study, only the first positive serum sample was used to determine whether Q fever showed a seasonal distribution and to evaluate the incidence of Q fever. For each patient, geographic origin, sex, age, and test results were added to 1 database.

Every year, the NRC provides an activity report in which annual data are analyzed. For Q fever, the number of positive serum samples and the total number of serum tested are included. The report describes the number of acute and chronic Q fever cases for the year.

Q fever seasonality and geographic distribution during January 2000–December 2009 were analyzed. We also classified all patients during 2009 on the basis of 2 criteria: 1) first positive serologic result in 2009 and 2) clinical status of the patient in the database. This classification was performed to evaluate incidence of endocarditis according to serologic titer and modified Duke Criteria. Patients were classified as having definite or possible endocarditis (*11*).

Serologic Analysis

Antigens were prepared for testing of phase I and II samples as described (*10*). Serum samples were first screened at dilutions of 1:25, 1:50, and 1:100 by using an indirect immunofluroescent antibody (IFA) test. Serum samples positive for total antibodies against Q fever at 1:100 dilution were tested by IFA to determine the IgG, IgM, and IgA titers for phases I and II. Serum with a phase II IgG titer ≥200 and a phase II IgM titer ≥50 was predictive for acute Q fever. This serologic criterion was used to define acute Q fever. If a phase I IgG titer was ≥800, chronic Q fever was suspected, which depended on other clinical findings. A low level of IgG is considered evidence of past infection (*12*).

Statistical Analysis

Since 1991, serologic results have been entered into our laboratory database and can be extracted as Excel (Microsoft, Redmond, WA, USA) files. Excel sheets were analyzed by using PASW Statistics software version 17.0 (SPSS Inc., Chicago, IL, USA). Seasonality of acute Q fever during January 2000–December 2009 was studied by using Expert Modeler (SPSS Inc.). This software identified the best model to use to analyze the data. Dependent variable series were also analyzed by using Expert Modeler, which automatically generates the best-fitting seasonal or nonseasonal model. This software also provides a better understanding of the data and predictions for future points in the series. Stationary R^2 goodness was used to measure the level of compliance of the data compared with the theoretical model. The Ljung-Box test (http://www.itl.nist.gov/div898/software/dataplot/refman1/auxillar/ljungbox.htm) was used to test the null hypothesis that autocorrelations of the residual time series were equal to 0. Statistical analyses of geographic distribution, mean age, and sex were conducted by using Epi Info version 6 (Centers for Disease Control and Prevention, Atlanta, GA, USA).

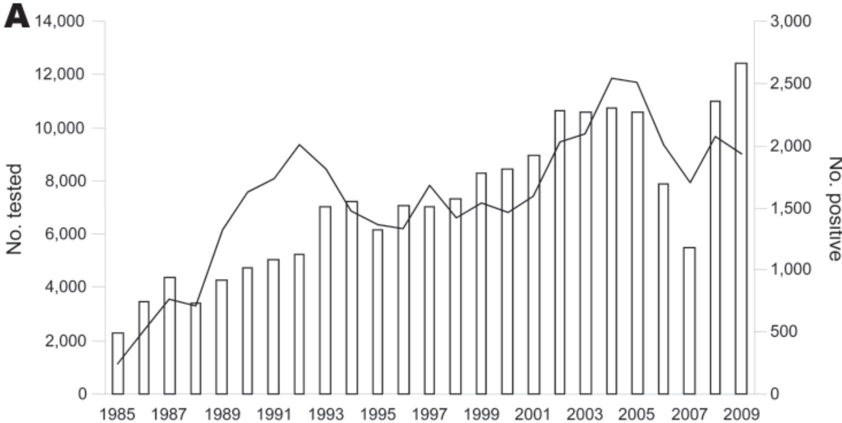
Evolution of Q Fever

During January 1985–December 2009, a total of 179,794 serum samples were analyzed (Table 1, Figure 1); 39,472 (30%) were positive, i.e., positive for antibodies at the first screening at a 1:100 dilution; 3,723 patients had acute Q fever (phase II IgG titer ≥200 and phase II IgM titer ≥50), and 1,675 had chronic Q fever (phase I IgG titer ≥800). The number of serum samples analyzed increased each year (except in 2006 and 2007), from 2,290 in 1985 to 12,443 in 2009. The number of positive serum samples also increased; peaks occurred in 1992 and in 2004. In 2004, the increase in serum samples positive for Q fever showed a

Table 1. Serum samples tested for Q fever, by year, France*

Year	No. positive/ no. tested (%)	No. positive	
		Acute disease	Chronic disease
1985	239/2,290 (10.4)	22	8
1986	518/3,464 (15.0)	37	8
1987	767/4,361 (17.6)	52	12
1988	706/3,403 (20.7)	49	12
1989	1,316/4,258 (30.9)	40	18
1990	1,621/4,720 (34.3)	92	22
1991	1,737/5,028 (34.5)	112	20
1992	2,011/5,249 (38.3)	89	29
1993	1,815/7,020 (25.9)	76	26
1994	1,472/7,222 (20.4)	102	24
1995	1,359/6,171 (22.0)	73	34
1996	1,326/7,101 (18.7)	116	28
1997	1,683/7,050 (23.9)	70	35
1998	1,420/7,340 (19.3)	140	37
1999	1,537/8,296 (18.5)	199	32
2000	1,460/8,444 (17.3)	135	32
2001	1,589/8,974 (17.7)	167	38
2002	2,029/10,639 (19.1)	224	55
2003	2,094/10,588 (19.8)	242	52
2004	2,544/10,742 (23.7)	360	70
2005	2,514/10,597 23.7)	199	100
2006	2,010/7,891 (25.5)	266	237
2007	1,700/5,522 (30.8)	244	278
2008	2,073/10,981 (18.9)	256	263
2009	1,933/12,443 (15.5)	361	205
Total	39,472/179,794 (21.9)	3,723	1,675

*Acute disease, phase II (avirulent bacteria) immunoglobulin (Ig) G titer ≥200 and phase II IgM titer ≥50; chronic disease, phase I (virulent bacteria) IgG titer ≥800.



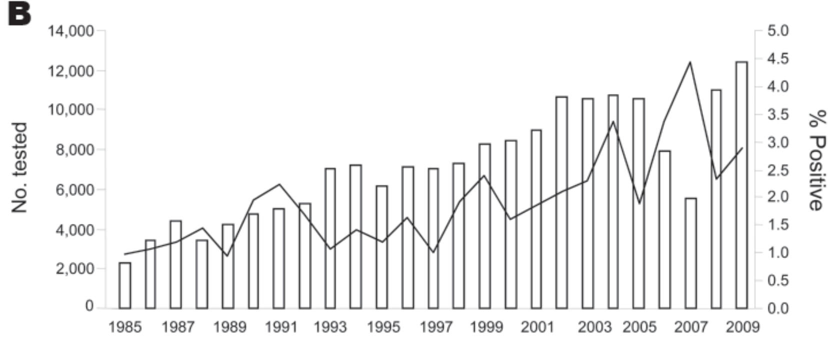


Figure 1. Serum samples tested for Q fever, France, 1985–2009. A) Black line indicates no. positive. B) Black line indicates % positive for acute Q fever.

correlation with increases in acute and chronic Q fever; 114 cases of acute Q fever were diagnosed in the Marseille area. Of the patients with acute Q fever, we had data for 919 (33.8%) female patients and 1,799 (66.2%) male patients.

Cases of acute Q fever increased continuously during 1985–2009 (Figure 2). The number of patients with serologic results for Q fever was stable; <100 cases were detected per year until 2004. Subsequently, >200 cases were diagnosed every year because physicians obtained more samples from patients for follow-up of acute infection (Figure 2).

The monthly distribution of acute Q fever during January 2000–December 2009 was determined. Variations in number of acute Q fever cases periodically occurred (Figure 3). Infections peaks occurred every year during April–September. Lower infection rates occurred during October–January. No peaks were observed in 2003 or 2005, which had <30 cases per month. The highest value was observed in June 2009 and was related to the outbreak in Cholet and to cases in Bouches du Rhône.

The Autoregressive Integrated Moving Average model selected by the Expert Modeler found a good correlation between observed and predicted values and also demonstrated an ability to predict peaks of infections. The stationary R^2 goodness-of-fit obtained with this model indicated that 49% of the variation in the number of acute Q fever cases was explained by the model (i.e., the month, which is the variable in our model, explained 49% of the fluctuation in the rate of Q fever rate). However, the Ljung-Box test value of 0.034 suggested that patterns in the observed series were not accounted for in the model.

Data regarding geographic distribution were obtained during January 1, 2000–December 31, 2009 (Figure 4). Geographic origin was known for 2,048 of 2,798 patients; data for 750 (26%) patients were missing. A total of 907 patients with Q fever were living in Provence Alpes Côte d'Azur, the region in which the NRC is located. The incidence of Q fever in this region was 19 cases/1 million inhabitants/y. Rhône-Alpes and Poitou-Charentes each had 12.7% of the cases. Incidence of cases in other regions did not exceed 6% (Figure 4).

Patient age was recorded for 2,658 patients during 2000–2009. The mean ± SD age was 46.2 ± 16.8 years, and the median age was 45 years (range 0–93 years). The mean ± SD age was 44.9 ± 18 years (median 43 years, range <1–90 years) for female patients and 46.8 ± 16.14 years (median 46 years, range 1–93 years) for male patients. The difference in mean age between male and female patients was significant (p = 0.0072).

In 2009, we obtained clinical information for 325 patients with positive serologic results. For this study, we classified the patient only if we obtained echocardiographic results for each patient. This process resulted in data for 172 patients according to the modified Duke Criteria (*11*).

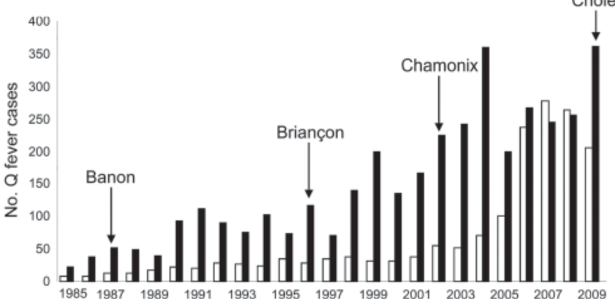


Figure 2. Cases of chronic (white bars) and acute (black bars) Q fever, France, 1985–2009. Locations where outbreaks were reported are indicated by arrows.

We classified these 172 patients according to their phase I IgG titers and identified 4 possible cases of endocarditis and 0 definite cases of endocarditis in patients whose phase I IgG titers were <800. For patients with phase I IgG titers ≥800, 1,600, 3,200 and 6,400, the numbers of definite cases of endocarditis were 41, 32, 24, and 17, respectively, and the numbers of possible cases of endocarditis were 11, 9, 3, and 1, respectively. The positive predictive values (PPVs) of IgG1 serologic results for possible or definite endocarditis were 37%, 59%, 57%, and 75% at titers of 800, 1,600, 3,200 and ≥6,400, respectively (Table 2).

Reemergence?

In this study, we demonstrated an increase in the yearly incidence of acute Q fever. Although we observed a clear trend of increased testing (Table 1), the data also showed an increase in the percentage positive among the samples being tested, increasing from 1% in 1989 to 3%–4% in 2005–2009 (Figure 1). These findings suggest several interpretations of this increased incidence. One interpretation could be improved diagnostic capability caused by development and availability of commercial diagnostic tests. At this stage, only ELISAs (Panbio, Sinnamon Park, Queensland, Australia, and Virion/Serion, Würzburg, Germany) and immunofluorescent assays (Focus Diagnostics, Cypress, CA, USA) are available. In our laboratory, in addition to PCR, an in-house method based on an IFA has been used for Q fever diagnosis since 1985. The continuity of our techniques enables comparison of results. Ake et al. demonstrated heterogeneous serologic results that depended on the method used (*13*). They focused on the necessity of having a reference laboratory to reduce potential interlaboratory and interassay variability.

Despite the increasing number of serum samples received by the NRC, our collection was insufficient to accurately determine the incidence of Q fever in France. In the United States, surveillance for this disease has historically been limited, and only 436 cases were reported during 1978–1999. In 1999, Q fever became a reportable disease in the United States, and the number of cases increased by 250% during 2000–2004 because of improved recognition and reporting (*14*). This study shows the need for national surveillance and confirms that lack of interest in the disease has consequences when incidence is determined.

Outbreaks of Q fever in France contributed to increases in incidence in 1987, 1996, 2002–2003, and 2009. However, incidence can be determined only if physicians are aware of Q fever. In 1987, forty cases of acute Q fever were diagnosed at a psychiatric institution in Banon, France (*15*). This outbreak affected patients and staff who worked on a farm where goats were raised for milk and cheese production. An outbreak was also reported in 1996 in Briançon, France, where 29 cases of acute Q fever were identified (*16*). Analysis of risk factors in a case–control study suggested that transmission resulted from airborne contaminated sheep waste, which had been left uncovered in a slaughterhouse area. Another outbreak was detected in 2002 in Chamonix, France, in which 101 patients were diagnosed with acute Q fever during August 30, 2002–July 31, 2003 (*17*). The most recent outbreak in France was at an abattoir in Cholet in 2009, in which 50 cases of acute Q fever were detected and confirmed (D. Raoult, unpub. data).

Outbreaks in the Netherlands during the past 3 years (182, 1,000, and 2,361 cases in 2007, 2008, and 2009, respectively) have emphasized the need to detect Q fever in livestock, particularly in goats (*4*). When infected animal fetuses are aborted, massive amounts of microbes are released. Goats and other infected animals are predominantly asymptomatic, which makes Q fever difficult to detect. Investigations in the Netherlands showed that outbreaks in 2007, 2008, and 2009 started in ≈2005. Measures taken in this country included massive vaccination of goats with an animal vaccine produced by CEVA (Libourne, France). Although this vaccine does not prevent all infections and is not effective in infected

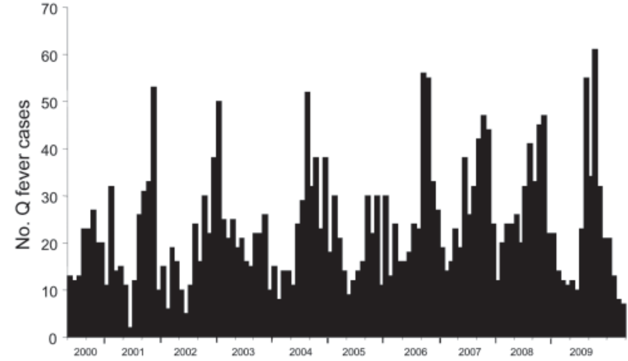


Figure 3. Seasonality of acute Q fever cases, France, 2000–2009.

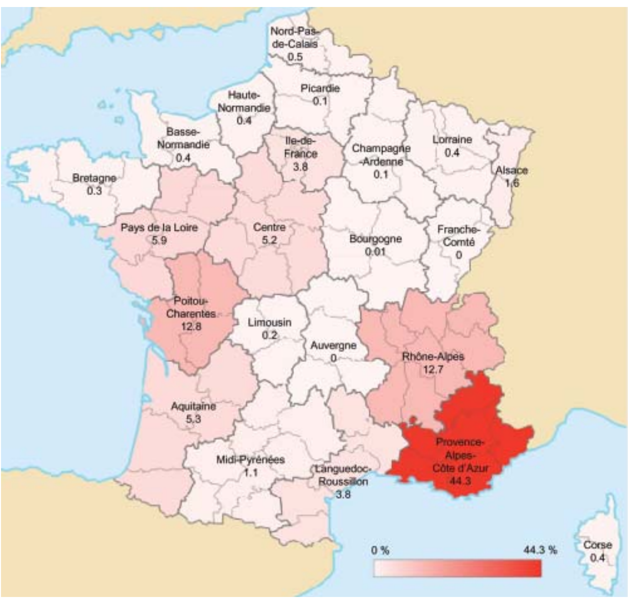


Figure 4. Geographic distribution of acute Q fever cases, France, 2000–2009. Values and scale bar indicate % prevalence.

animals, it does prevent most abortions. Therefore before vaccination, farmers must kill all pregnant goats on affected farms, regardless of the vaccination status of the animals. However, although outbreaks were a factor in the increase in Q fever, they were likely not the sole cause of increases in recent years.

Another reason for the increase in incidence of Q fever was increased interest of physicians in this disease. Results from a survey showed that the highest incidence of this disease in France was in Bouches du Rhône, where our laboratory is located. The proximity of the NRC has been suggested to influence the number of cases detected in this area; the annual incidence in our area (19 cases/1 million persons) was much higher than in the rest of France (3 cases/1 million persons). However, other reasons may explain changes in the geographic distribution of this disease.

Van der Hoek et al. reported the distribution of Q fever in humans in the Netherlands in 2009; a total of 59% of the cases were in persons who lived within a 5-km radius of an infected dairy sheep or dairy goat farm (12% of the population lived in these areas). The incidence was 69 cases per 100,000 persons in those who lived within a 5-km radius of infected livestock and 6 cases per 100,000 persons in those who lived outside the 5-km radius (*18*). Angelakis and Raoult reported that contaminated aerosols are the major mechanism for transmission of *C. burnetii* to humans (*19*). Data for 2008 from the French Agriculture Ministry (www.agreste.agriculture.gouv.fr) indicate that regions with the highest incidence of acute Q fever are regions with the highest number of cattle, sheep, and goats. Provence Alpes Côte d'Azur, the region in which the largest number of cases was detected, ranks fourteenth among French regions for number of livestock. Rhône-Alpes and Poitou Charente rank twelfth and sixth, respectively. Midi-Pyrénées, which contains the largest number of livestock, accounted for only 1.1% of acute Q fever cases in France.

Analysis of the monthly distribution of acute Q fever showed seasonality of the disease. Several factors might account for this phenomenon. *C. burnetii* is present in aerosols of parturient fluids from infected animals (*1*). Tissot-Dupont et al. reported seasonal increases in April and June (*20*) that were not correlated with sheep birth, which occurs in October. This observation could be explained by an increased number of lambs killed for Easter. Tissot-Dupont et al. also reported the role of wind in transmitting *C. burnetii* (*7*); an increase in incidence of Q fever during winter of 1998–99 correlated with increased velocity of mistral (the wind in southern France). These authors reported fewer cases of Q fever occurred in mountainous areas than in plains (*20*). A study in the United Kingdom demonstrated the role of airborne transmission (*21*). Hellenbrand et al. analyzed cases of Q fever in Germany during 1962–1999 (*22*). They found an irregular cyclic incidence pattern, but did not distinguish between acute and chronic forms of the disease. They also reported a change in community outbreak seasonality in Germany: a marked decrease in winter and an increase in summer. These changes coincide with decreases in nomadic sheep farming. These authors concluded that the incidence of Q fever has increased in Germany.

The number of patients in France with a chronic Q fever serologic profile has increased since 2006. However, with an average of 67 cases of chronic Q fever detected per year during 1985–2009, we expected to identify 50% of the chronic disease cases in France. Our data are more

Table 2. PPV of Q fever phase I IgG titers for patients with endocarditis, France, 2009*

	Titers			
Characteristic	≥800	≥1,600	≥3,200	≥6,400
No. with definite endocarditis	41	32	24	17
No. with possible endocarditis	11	9	3	1
Total	141	88	47	24
PPV, % (definite or possible endocarditis)	37	59	57	75

*PPV, positive predictive value; phase I, virulent bacteria; Ig, immunoglobulin.

comprehensive because the NRC is the only laboratory that analyzes the phase I antibodies against Q fever in France.

Criteria for diagnosis of chronic Q fever were established in 1994 (*10*). Before this study, the PPV was determined by using Bayes theorem, and patients with endocarditis were tested for Q fever. The PPV for phase I IgG titers of 800 and 1,600 was high (98.1% and 100%, respectively). When we analyzed data from 2009, the PPV for possible or definite Q fever endocarditis (Table 2) was 37% for patients with phase I IgG titers ≥800 and 75% for patients with phase I IgG titers ≥6,400. This difference between the 2 studies reflects the increasing number of samples tested. In 2009, we diagnosed 41 definite and 11 possible cases of endocarditis caused by *C. burnetii*. A study published in 2000 with NRC data for 1985–1998 reported 259 cases of Q fever endocarditis and a mean of 20 cases per year (*3*).

The serum collection at the NRC is more comprehensive than it was 10 years ago, which could explain the increase in the number of cases. We believe that the number of *C. burnetii* endocarditis cases diagnosed at the NRC represents 50%–80% of the Q fever endocarditis cases in France. These data confirm previous data from which the prevalence of Q fever endocarditis in the general population in France was estimated to be 1 case/1 million persons/y. Several studies have estimated that 3%–5% of symptomatic *C. burnetii* infections cause endocarditis (*1*). These data enabled us to estimate the minimum incidence of mild-to-severe acute Q fever to be 20 cases/1 million persons/y, which is similar to that observed in Provence-Alpes-Côte d'Azur (19 cases/1 million persons/y) but lower than that observed in Bouches du Rhône (40 cases/1 million persons/y in 2009). When no outbreak is observed, the incidence of cases diagnosed in France at the NRC reflects the true incidence of acute infections.

Q fever has been recognized since the 1930s. We believe that its increased frequency in recent years is a combination of an increase in the disease (reemergence), growing interest among physicians, better diagnosis in laboratories, and more complete data. Monthly analysis of the past decade demonstrates the seasonality of Q fever, which shows peaks during April–September in Europe. We propose a change in the phase I IgG cutoff titer for detection of Q fever endocarditis to ≥1,600; this change corresponds to a PPV of 59%. This adjusted cutoff value may increase detection of endocarditis in patients with a diagnosis of chronic Q fever. Finally, the number of identified epidemics in France and Europe is increasing. Increased surveillance of the disease is the only way to determine the effects of Q fever.

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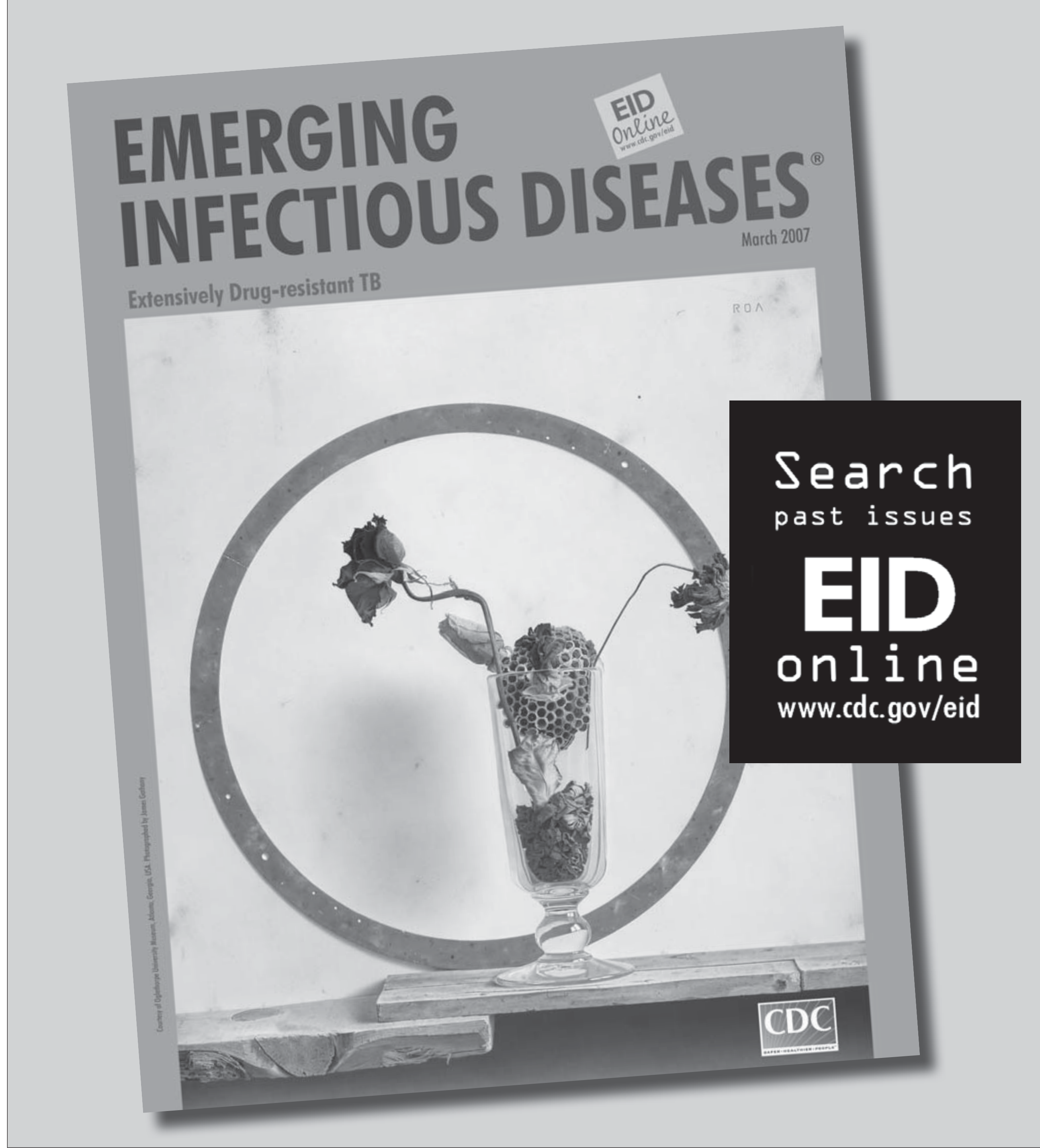

Active Tuberculosis among Homeless Persons, Toronto, Ontario, Canada, 1998–2007

Kamran Khan, Elizabeth Rea, Cameron McDermaid, Rebecca Stuart, Catharine Chambers, Jun Wang, Angie Chan, Michael Gardam, Frances Jamieson, Jae Yang, and Stephen W. Hwang

While tuberculosis (TB) in Canadian cities is increasingly affecting foreign-born persons, homeless persons remain at high risk. To assess trends in TB, we studied all homeless persons in Toronto who had a diagnosis of active TB during 1998–2007. We compared Canada-born and foreign-born homeless persons and assessed changes over time. We identified 91 homeless persons with active TB; they typically had highly contagious, advanced disease, and 19% died within 12 months of diagnosis. The proportion of homeless persons who were foreign-born increased from 24% in 1998–2002 to 39% in 2003–2007. Among foreign-born homeless persons with TB, 56% of infections were caused by strains not known to circulate among homeless persons in Toronto. Only 2% of infections were resistant to first-line TB medications. The rise in foreign-born homeless persons with TB strains likely acquired overseas suggests that the risk for drug-resistant strains entering the homeless shelter system may be escalating.

In Canada's major cities, tuberculosis (TB) is increasingly becoming a disease of persons born outside Canada (foreign-born). In 2009 in the city of Toronto in Ontario, 94% of all persons with active TB were foreign-born (*1*). Although homeless and marginally housed persons represent a smaller proportion of TB case-patients, they remain a persistent high-risk population. Recent TB outbreaks and disease clusters among homeless persons have been reported in many cities in the United States (*2–5*) and have been associated with transmission at shelters, single-room–occupancy hotels, and rooming houses (which provide inexpensive rooms with shared bathrooms), prisons, and bars (*6–10*).

Toronto is the largest city in Canada; among its population of 2.5 million persons, ≈50% were born outside Canada (*11*). Each year in Toronto, ≈29,000 persons use emergency shelters, and on any given night ≈5,000 are without homes (*12*). During 2001–2002, a large shelter-based TB outbreak occurred among homeless persons in Toronto. A coroner's inquest into the death of a homeless man in whom pulmonary TB developed during the course of this outbreak revealed the many challenges of diagnosing and managing TB in homeless populations (*13*). In response to the inquest and resulting jury recommendations, major changes to the management of homeless TB cases occurred in the public health and shelter systems, and local TB clinic capacity expanded. This case resulted in the creation of a public health team dedicated to case management, contact follow-up, advocacy, education, health promotion, and active case finding among the city's homeless and underhoused population.

A comprehensive review of the population and molecular epidemiology, clinical features, management and health outcomes of homeless persons with TB in Canada is needed but lacking. To better understand and address the extent of disease in this vulnerable population, we studied TB among Toronto's homeless persons over a 10-year period.

Methods

The study population included all persons in Toronto for whom active TB had been reported to Toronto Public Health from January 1, 1998, through December 31,

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2007. Data were extracted from the Reportable Disease Information System and the Integrated Public Health Information System for all case-patients with a risk setting of "shelter/rooming house" or a risk factor of "homeless." Health case management files were reviewed to ensure accuracy of database entries; additional data were abstracted when necessary. Cases were included in the analysis for persons with active TB who met the following eligibility criteria in the year before diagnosis: 1) any shelter stay, 2) any rooming house stay, 3) no fixed address, or 4) use of services for homeless persons >1× per week. Cases were excluded for persons with active TB who 1) were foreign-born and received a diagnosis of active TB within 1 month of arrival in Canada, 2) received a diagnosis of active TB while in a shelter designed exclusively for resettlement of newly arrived refugees, 3) were not residents of Toronto when they received a diagnosis of active TB, or 4) had incomplete records.

We collected data on patient demographics, clinical features of TB disease, medical management and health outcomes of patients, and molecular fingerprinting of the TB bacterium. Case types were classified according to the Public Health Agency of Canada definition of new and re-treatment TB cases (*14*). All chest radiographs were interpreted by radiologists. Susceptibility testing for *Mycobacterium tuberculosis* was performed at the Central Public Health Laboratory of the Ontario Agency for Health Protection and Promotion. All isolates from new TB cases were tested for susceptibility to first-line drugs (isoniazid, rifampin, pyrazinamide, and ethambutol) according to recommended standard protocols, by using the commercial broth system, BACTEC MGIT 960 (Becton, Dickinson and Company, Sparks, MD, USA). Isolates resistant to rifampin or any 2 first-line drugs were also tested for susceptibility to second-line drugs (*15*). Restriction fragment-length polymorphism was performed for strain genotyping by using established methods (*16*). Genotypes were analyzed by using Bionumerics 5.0 (Applied Maths, Saint-Martens Latem, Belgium). HIV test results were recorded when available; information about use of antiretroviral therapy for HIV/TB–co-infected patients was not available.

Comparisons were made between Canada-born and foreign-born case-patients and between 5-year periods (1998–2002 and 2003–2007) using the 2-sided Fisher exact test or χ^2 test, as appropriate. Kaplan-Meier plots were generated to determine time to death from all causes during the 12 months after TB diagnosis. The log-rank test was used to compare survival curves between the 2 groups. Because of the small cohort size, multivariate regression analyses were not performed. All analyses were performed by using SAS version 9.1.3 (SAS Institute, Cary, NC, USA). Ethics approval was obtained from St. Michael's Hospital Research Ethics Board.

Results

From January 1, 1998, through December 31, 2007, a total of 3,685 active TB cases were reported to Toronto Public Health; among these, 102 (2.8%) met the study inclusion criteria. Incomplete records for 11 patients resulted in a final sample size of 91 (Figure 1). Most patients were absolutely homeless (i.e., living on the street or in a shelter); 86 (95%) patients reported staying in a shelter, having no fixed address, and/or using services for homeless persons >1× per week. Five (5%) patients did not fall into any of these categories but had lived in a rooming house during the past year. Birthplace was available for 88 patients; nearly one third (n = 28; 32%) were born outside Canada (Table 1). The proportion of foreign-born patients increased over time from 24% (n = 10) in 1998–2002 to 39% (n = 18) in 2003–2007 (Table 2). Among the Canada-born homeless persons with TB, 13 (22%) were Aboriginal. The number of reported cases of active TB over the study period by place of birth is shown in Figure 2. Approximately equal numbers of cases were reported during the 2 periods: 44 (48%) during 1998–2002 and 47 (52%) during 2003–2007.

Demographic information, clinical characteristics, and concurrent medical conditions for patients are presented in Tables 2 and 3. Homeless persons with TB were often highly contagious at the time of diagnosis, as demonstrated by the large proportion of patients who had cavitating pulmonary disease and sputum smears with numerous acid-fast bacilli. The median duration of symptoms for persons with pulmonary disease before diagnosis was 2.5 months (interquartile range 0.6–3.1 months). Pulmonary disease was found in 67 (74%) patients, among whom 29 (46%) showed numerous acid-fast bacilli in sputum smear. The proportion of pulmonary TB patients with cavitary disease

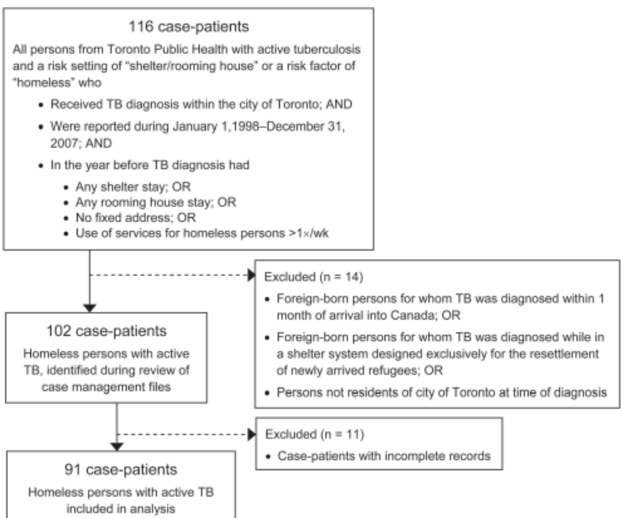

Figure 1. Inclusion–exclusion criteria for study of active tuberculosis (TB) in homeless persons, Toronto, Ontario, Canada, 1998–2007.

Table 1. Country of origin for 28 foreign-born homeless persons with tuberculosis, Toronto, Ontario, Canada, 1998–2007

Country	No.
Burundi	1
Chile	1
China*	1
Costa Rica	1
Ethiopia*	2
Germany	1
Guyana	2
India*	2
Iraq	1
Ireland	2
Nepal	1
Nigeria*	1
Philippines*	1
Poland	1
Somalia	2
Tanzania*	1
Tibet	1
Turkey	1
Uganda*	2
United Kingdom	1
Yemen	1
Former Yugoslavia	1
Zimbabwe*	2

*1 of 22 countries that account for 80% of all new of tuberculosis cases annually (high-burden countries).

increased over time from 14% (n = 5) in 1998–2002 to 32% (n = 11) in 2003–2007.

In terms of treatment information and outcomes, 75% of homeless persons with TB started treatment within 4 days of diagnosis (median 1 day; interquartile range 0–4 days) (Table 4). Most patients received closely monitored treatment within hospitals or as outpatients under directly observed therapy (DOT) (median treatment duration 2.0 and 6.2 months, respectively); few received self-administered therapy for any substantial period of time. Only 1 patient required a court order for treatment in a TB sanitarium.

Almost 1 of 5 (n = 17; 19%) patients died (from any cause) within 12 months of diagnosis (Table 4); most (n = 12; 86%) patients who died were born in Canada; 4 were HIV positive, 1 was HIV negative, and the remaining 12 had unknown HIV status. Among patients who survived, most (n = 70; 96%) homeless persons with TB successfully completed treatment and only 3 were lost to follow-up or refused further care. Probability of survival during the 12 months after diagnosis was lower for Canada-born versus foreign-born homeless persons (p = 0.06; Figure 3). No changes in survival probabilities were seen between the 2 periods, 1998–2002 and 2003–2007 (data not shown).

TB isolates were genotyped and tested for antimicrobial drug resistance (Table 5). Of the 4 strains known to circulate among Toronto's homeless population (A, B, C, or D), isolates from ≈90% of Canada-born patients belonged to one of these strains, and isolates from >50% of foreign-born patients belonged to none of them. The proportion of isolates not belonging to these 4 strains increased over time, from 14% (n = 4) during 1998–2002 to 32% (n = 12) during 2003–2007. Almost all (n = 84; 98%) isolates were susceptible to first-line TB medications. Only 2 isolates demonstrated evidence of antimicrobial drug resistance: 1, from a Canada-born patient, was resistant to ethambutol only, and 1, from a foreign-born patient, was resistant to isoniazid only.

Discussion

Homeless persons in our cohort received nearly all health care services for TB in hospital or under careful observation in the outpatient environment. All outpatients received DOT, were accompanied by public health staff to all clinic visits, and received intensive social assistance. Despite these efforts, all-cause mortality rates for our cohort were extremely high. Among Canada-born homeless persons with TB in our study, 20% died within 1 year of their diagnosis; in comparison, among all persons with TB in Toronto during 1999–2002, only 7.4% died (*17*). All-cause mortality rates for homeless populations in general are disproportionately high; rates among men who use shelters in Toronto are 2–8× higher than rates for the general population (*18*). Homeless persons often have more concurrent medical conditions (e.g., HIV, liver disease), mental health conditions (e.g., schizophrenia), and/or dependence on substances, any of which may raise their risk for primary or reactivated TB, complicate delivery of health services, and negatively affect treatment outcomes.

Our findings reflect the increased rates of illness and death among homeless persons and suggest that urgent measures are needed to improve TB treatment outcomes for this vulnerable population. Recent research suggests that homeless immigrants in Toronto are in general healthier and possess fewer concurrent illnesses than Canada-born homeless persons, which may explain the lower prevalence of concurrent illnesses among foreign-born TB patients and the differences in mortality rates according to place of birth (*19*).

Homeless TB patients represent ≈3% of all TB patients in Toronto, of which a growing proportion are foreign-born, likely reflecting the changing demographics in the city overall and in the homeless population itself. Our findings suggest that Canada-born patients with TB were more likely to be infected with strains known to circulate within shelters and other social networks in Toronto. In contrast, active TB in foreign-born patients was more likely to result from reactivation of latent infection with strains acquired overseas (*20,21*).

To date, drug resistance among homeless TB patients is rare; laboratory evidence of drug resistance

Table 2. Demographic and clinical characteristics of 91 homeless persons with tuberculosis, Toronto, Ontario, Canada, 1998–2007*

Characteristic	All persons, no. (%)	Canada born, no. (%)	Foreign born, no. (%)	p value†	1998–2002, no. (%)	2003–2007, no. (%)	p value‡
Median age, y (IQR)	47 (38–56)	49 (42–58)	38 (30–50)	–	45 (38–59)	48 (40–54)	–
Male sex	81 (89)	53 (88)	25 (89)	1.00	40 (91)	41 (87)	0.74
Origin							0.15
Canada born, not Aboriginal	47 (53)	47 (78)	NA		27 (64)	20 (43)	
Canada born, Aboriginal	13 (15)	13 (22)	NA		5 (12)	8 (17)	
Foreign born	28 (32)	NA	28 (100)		10 (24)	18 (39)	
Case type				0.43			1.00
New	83 (91)	53 (88)	27 (96)		40 (91)	43 (91)	
Re-treated§	8 (9)	7 (12)	1 (4)		4 (9)	4 (9)	
Method of detection				–			–
Signs and symptoms	51 (56)	30 (50)	18 (64)		23 (52)	28 (60)	
Contact tracing	19 (21)	16 (27)	3 (11)		11 (25)	8 (17)	
Diagnosis while under care for other condition	8 (9)	8 (13)	0		4 (9)	4 (9)	
Immigration screening	6 (7)	NA	6 (21)		6 (14)	0	
Active case finding (sputum screening)	3 (3)	3 (5)	0		NA	3 (6)	
Jail	1 (1)	1 (2)	0		0	1 (2)	
Other¶	3 (3)	2 (3)	1 (4)		0	3 (6)	
Site(s) of infection				1.00			0.15
Pulmonary only	67 (74)	45 (75)	21 (75)		33 (75)	34 (72)	
Extrapulmonary only	21 (23)	13 (22)	6 (21)		8 (18)	13 (28)	
Pulmonary and extrapulmonary	3 (3)	2 (3)	1 (4)		3 (7)	0	
Chest radiograph at diagnosis#							0.08
No abnormalities	9 (13)	7 (15)	2 (9)	0.86	7 (19)	2 (5)	
Abnormal without cavitation	45 (65)	30 (64)	15 (68)		24 (67)	21 (62)	
Abnormal with cavitation	15 (22)	10 (21)	5 (23)		5 (14)	11 (32)	
Self-reported symptoms	73 (80)	50 (83)	20 (71)	0.26	34 (77)	39 (83)	0.60
Median time from symptom onset to diagnosis, mo (IQR)	1.9 (0.6–3.1)	1.8 (0.6–2.6)	2.6 (0.8–5.8)	–	1.8 (0.6–3.1)	2.2 (0.6–3.2)	–
Sputum smear results at diagnosis**				0.39			0.58
Negative	21 (33)	12 (29)	9 (43)		12 (40)	9 (27)	
Scarce/moderate	13 (21)	8 (20)	5 (24)		5 (17)	8 (24)	
Numerous	29 (46)	21 (51)	7 (33)		13 (43)	16 (49)	
Method of diagnosis				0.59			0.18
Positive culture	86 (95)	55 (92)	28 (100)		40 (91)	46 (98)	
Positive AMTD	3 (3)	3 (5)	0		3 (7)	0	
Clinical	2 (2)	2 (3)	0		1 (2)	1 (2)	

*Birthplace information available for 88 persons. IQR, interquartile range; NA, not applicable; –, no statistical test performed; AMTD, amplified *Mycobacterium tuberculosis* direct test.
†Probability of a significant difference between Canada-born and foreign-born persons for each variable; calculated by using the 2-sided Fisher exact test or χ^2 test, as appropriate.
‡Probability of a significant difference between the 2 periods for each variable; calculated by using the 2-sided Fisher exact test or χ^2 test, as appropriate.
§Public Health Agency of Canada definition: documented evidence or adequate history of previously active tuberculosis (TB) that was declared cured or treatment completed by current standards, AND at least 6 mo have passed since the last day of previous treatment, AND a diagnosis with a subsequent episode of TB that meets the active TB case definition.
¶Includes shelter screening, routine screening at other centers, and other detection methods.
#Results for only the 70 persons with pulmonary disease.
**Sputum smears available for 63 patients with pulmonary disease.

was demonstrated for only 2% of homeless TB patients compared with 14% of all culture-positive TB patients in Toronto (*1*). Because being born outside Canada is a known risk factor for drug-resistant TB, the rise in foreign-born homeless TB patients and the corresponding increase in heterogeneity of strain genotypes are concerning and may pose serious and growing threats to the homeless shelter system (*20,22–25*). The outbreaks of multidrug resistant TB in New York City during the 1980s and early 1990s highlight the potential dangers of introducing drug-resistant infections into the shelter system and call for increased prevention and control efforts (*26*).

Despite the increase in foreign-born homeless persons with TB over time, most homeless TB patients in our sample were Canada-born (68%), a substantial proportion of whom were of Aboriginal origin (22%). By comparison,

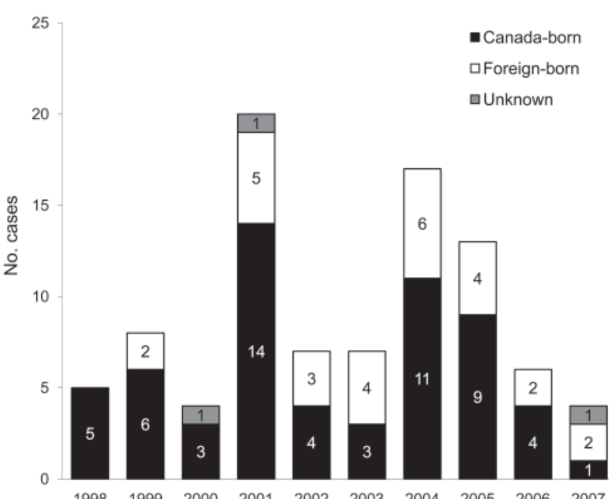

Figure 2. Number of reported cases of active tuberculosis in homeless persons, Toronto, Ontario, Canada, 1998–2007.

in 2008, only 6% of persons with active TB in Toronto were Canada-born (*1*). Although TB in Canada is primarily a disease of foreign-born persons (*14*), our results suggest that TB transmission persists among Canada-born inner city homeless populations. These findings also underscore the need to address TB transmission within the homeless shelter system. Furthermore, the disproportionately high prevalence of Canada-born Aboriginal persons in our sample suggests that further efforts are needed to address the high incidence of TB in this population.

Homeless TB patients tend to seek care when disease is advanced and highly contagious, defined by abnormal chest radiographic findings (cavitation) and numerous acid-fast bacilli in sputum smear. Although many homeless patients had pulmonary disease, the number was proportional to the prevalence of pulmonary disease among all TB patients in Toronto (*1*). In patients with pulmonary TB, nearly half had numerous acid-fast bacilli in sputum smear. An increasing prevalence of cavitary disease on chest radiographs was observed over time, despite increasing intervention and active case-finding initiatives during the more recent 5-year period of this study. However, the increase in cavitary disease could be related to delays in seeking health care, as indicated by the increase in median time from symptom onset to diagnosis over the 2 periods of the study.

Homeless TB patients often have difficulty accessing the health care system and may prioritize subsistence needs such as food and shelter over health services, especially those perceived as discretionary (*27*). These factors, as well as cultural and language barriers among foreign-born patients (*28,29*), may contribute to delays in seeking health care, which lead to advanced disease and hospitalization (*27,30,31*). For our sample, hospitalization rates were high; >80% of patients were hospitalized. This is noteworthy in Canada, where most TB patients are treated as outpatients, even at the time of diagnosis (*32*). The inability to isolate infectious homeless patients in outpatient settings such as shelters largely explains the high rate of hospitalization for patients in our sample.

Adherence to treatment is often challenging for patients who are homeless or living in transient, substandard housing and who may have concurrent substance use or mental health problems. Consequently, DOT is usually implemented for homeless persons with TB in Canada (*32*). In the province of Ontario, all patients with active

Table 3. Concurrent conditions of 91 homeless persons with tuberculosis, Toronto, Ontario, Canada, 1998–2007*

Condition	All persons, no. (%)	Canada born, no. (%)	Foreign born, no. (%)	p value†	1998–2002, no. (%)	2003–2007, no. (%)	p value‡
HIV infection				0.52			0.33
Positive	11 (12)	9 (15)	1 (4)		5 (11)	6 (13)	
Negative	6 (7)	4 (7)	2 (7)		1 (2)	5 (10)	
Unknown	74 (81)	47 (78)	25 (89)		38 (87)	36 (77)	
Psychiatric disease§	10 (11)	7 (12)	2 (7)	0.71	2 (5)	8 (17)	0.09
COPD	8 (9)	7 (12)	0	0.09	6 (14)	2 (4)	0.15
Liver disease¶	29 (32)	24 (40)	4 (14)	0.03	12 (27)	17 (36)	0.38
Cancer	5 (5)	4 (7)	1 (4)	1.00	3 (7)	2 (4)	0.67
Congestive heart failure	1 (1)	0	0	–	1 (2)	0	0.48
Diabetes	11 (12)	5 (8)	5 (18)	0.28	1 (2)	10 (21)	0.01
Chronic alcohol abuse	29 (32)	23 (38)	6 (21)	0.15	12 (27)	17 (36)	0.38
Injection drug use	12 (13)	10 (17)	1 (4)	0.16	6 (14)	6 (13)	1.00
Noninjection drug use	6 (7)	6 (10)	0	0.17	0	6 (13)	0.03
Other	1 (1)	0	1(4)	0.32	0	1 (2)	1.00

*Birthplace information available for 88 persons. –, no statistical test performed; COPD, chronic obstructive pulmonary disease.
†Probability of a significant difference between Canada-born and foreign-born persons for each variable; calculated by using the 2-sided Fisher exact test or χ^2 test, as appropriate.
‡Probability of a significant difference between the 2 periods for each variable; calculated by using the 2-sided Fisher exact test or χ^2 test, as appropriate.
§Includes schizophrenia, severe mental illness, and dementia.
¶Includes cirrhosis, viral hepatitis B or C.

Table 4. Treatment-associated characteristics of 91 homeless persons with tuberculosis, Toronto, Ontario, Canada, 1998–2007*

Characteristic	All persons, no. (%)	Canada-born, no. (%)	Foreign born, no. (%)	p value†	1998–2002, no. (%)	2003–2007, no. (%)	p value‡
Days from diagnosis to initiation of treatment, median (IQR)	1 (0–4)	0.5 (0–4)	1 (0–6)	–	1 (0–3)	1 (0–4)	–
Duration of treatment, mo, median (IQR)							
Total	9.9 (6.5–12.7)	10.0 (6.8–12.5)	8.6 (6.0–12.9)	–	9.0 (6.1–12.2)	10.8 (6.7–13.1)	–
Treatment in institution	2.0 (0.2–4.0)	2.4 (0.5–3.9)	0.6 (0–4.2)		0.9 (0.2–3.9)	2.2 (0.0–4.1)	
Treatment under directly observed therapy	6.2 (0.8–8.8)	6.4 (0.9–9.0)	6.0 (2.4–7.2)		5.7 (0.2–7.0)	6.6 (4.5–9.0)	
Treatment by self-administered therapy	0.1 (0.1–0.9)	0.1 (0.1–0.6)	0.3 (0.1–2.1)		0.2 (0.0–1.1)	0.1 (0.1–0.6)	
Admission to hospital	76 (84)	55 (92)	19 (68)	0.01	35 (80)	41 (87)	0.40
Treatment under public health order§	15 (16)	9 (15)	6 (21)	0.55	4 (9)	11 (23)	0.09
Court-ordered detention for treatment¶	1 (1)	1 (2)	0	–	1 (2)	0	–
Outcome#				0.13			0.74
Treatment completed	70 (78)	45 (75)	25 (93)		33 (75)	37 (80)	
Died while receiving treatment**	17 (19)	12 (20)	2 (7)		9 (20)	7 (16)	
Lost to follow-up	2 (2)	2 (3)	0		1 (2)	1 (2)	
Refused further care	1 (1)	1 (2)	0		1 (2)	0	

*Birthplace information available for 88 persons. IQR, interquartile range; –, no statistical test performed.
†Probability of a significant difference between Canada-born and foreign-born persons for each variable; calculated by using the 2-sided Fisher exact test or χ^2 test, as appropriate.
‡Probability of a significant difference between the 2 periods for each variable; calculated by using the 2-sided Fisher exact test or χ^2 test, as appropriate.
§Referred to in Ontario as a Section 22.
¶Referred to in Ontario as a Section 35.
#1 patient continues treatment at the time of this report.
**One foreign-born case-patient from 2003–2007 period died 14 mo after onset of treatment. Study considers death from all causes within 12 mo of TB diagnosis. After 12 mo, causes of death other than TB become more relevant; therefore, deaths occurring after 12 mo are not included in this estimate.

TB are eligible to receive either inpatient or outpatient TB treatment, regardless of their insurance coverage. Most patients in our sample received their entire treatment closely monitored within hospitals, with outpatient DOT, or both. A few patients self-administered treatment for short periods. Despite the common perception that homeless TB patients are noncompliant with treatment (*33–35*), ≈80% of patients in our sample completed treatment, which is equivalent to the treatment completion rate for all TB patients in Toronto receiving DOT (*1*). Intensive case management by public health and clinic staff as well as small incentives and enablers (e.g., food vouchers or cash) helped ensure high completion rates for this population. For most homeless TB patients who did not complete treatment, the reason was that they died; only a few were lost to follow-up or refused further care.

The strength of this study is that it provides a comprehensive description of all cases of TB among homeless persons in a large, ethnically diverse city in Canada over a 10-year period. However, the study also has limitations. Only patients who were residents of the city of Toronto at the time of diagnosis were included in the analysis; consequently, homeless persons with active TB who may have been exposed in Toronto shelters or rooming houses and later moved elsewhere were not detected. Furthermore, homeless persons with TB were not included in the analysis if they had a history of shelter use >1 year before diagnosis with active TB. As a result, some patients who acquired TB infection while homeless but who subsequently acquired housing may have been missed. Furthermore, our retrospective study used public health surveillance data; consequently, our analyses are subject to limitations in how the data were originally collected. We excluded 14 patients with active TB because their records were incomplete; hence, we were unable to determine whether they differed demographically from included patients. This limitation could have influenced the results of our analyses that were stratified by birthplace.

Molecular fingerprinting data were unavailable for 20 isolates, which may have influenced the genotyping trends we observed over time. Because we were unable to definitively determine the number of patients who died directly or indirectly as a result of TB, the mortality rates represent death from all causes in the year after TB diagnosis. Although our study was conducted in a single metropolitan urban center, our findings and recommendations may be relevant to other large cities where levels of immigration and poverty are high.

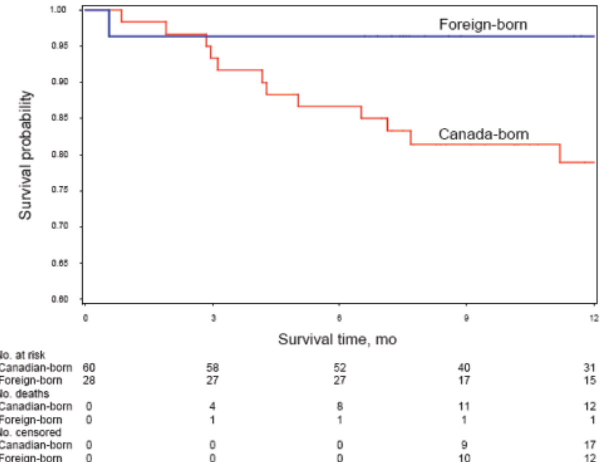

Figure 3. Probability of death from all causes during the 12-month period after tuberculosis diagnosis among Canada-born and foreign-born homeless persons with active tuberculosis, Toronto, Ontario, Canada, 1998–2007. Birthplace information available for 88 persons. Censored are patients who completed treatment for tuberculosis or were lost to follow up.

Prior research among homeless persons in New York City shows decreasing trends in rates of active TB during 1992–2006 and demonstrates that public health prevention and control efforts (e.g., latent TB infection screening) in this population can be effective (*36*). In our study, several homeless patients were originally identified as tuberculin skin test–positive contacts before active TB developed, but they were unwilling to start treatment for latent TB infection, were deemed poor candidates for treatment because of serious underlying medical or mental health conditions, or could not complete a course of treatment because of adverse drug reactions. Lack of treatment for latent TB occurred despite substantial incentives and enablers for persons to initiate and continue therapy (e.g., cash for attending clinic visits, free passes for taxis or public transit, use of DOT for latent TB infection). Hence, for this cohort the opportunity to mitigate the risk for development of active TB through the treatment of latent TB infection was limited by the above challenges.

Primary prevention efforts should focus on shelter-based control measures, which have proven effective at reducing person-to-person spread of drug-resistant TB in other urban centers (*37*). Improved ventilation systems at shelters will help reduce the spread of TB during an outbreak (*38,39*). Smaller shelter sizes and strategies to reduce mobility (e.g., eliminating length of stay restrictions at shelters) may also help limit the extent of transmission. Additionally, expansion of sustainable housing program for homeless and marginally housed populations will help reduce the number of persons needing to use shelters, subsequently decreasing the likelihood of TB exposure at these congregate settings.

Control of TB in homeless populations within Canada will require further progress in primary prevention (e.g., improved ventilation and other infection control measures in shelters), secondary prevention (e.g., earlier detection and treatment of TB infection or disease through greater access to primary care), and tertiary prevention (e.g., treatment of active TB by health care providers with experience treating TB in homeless persons) (*17*). Furthermore, Canada's interconnectedness with the global community, and consequent interdependence with global TB, necessitates continued vigilance to confront the emerging threat of drug-resistant TB in the world (*40*).

This study was supported by the Connor, Clark & Lunn Foundation, the St. Michael's Hospital Foundation, the Ontario Ministry of Health and Long-Term Care, and the Public Health Agency of Canada.

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Table 5. Characteristics of isolates from 91 homeless persons with tuberculosis, Toronto, Ontario, Canada, 1998–2007*

Characteristic	Culture-confirmed case, no. (%)	Canada-born, no. (%)	Foreign born, no. (%)	p value†	1998–2002, no. (%)	2003–2007, no. (%)	p value‡
RFLP type				<0.001			0.13
Strain A	32 (48)	25 (52)	6 (38)		17 (59)	15 (41)	
Strain B	8 (12)	7 (15)	1 (6)		3 (10)	5 (14)	
Strain C	7 (11)	7 (15)	0		5 (17)	2 (5)	
Strain D	3 (5)	3 (6)	0		0	3 (8)	
Other strain	16 (24)	6 (13)	9 (56)		4 (14)	12 (32)	
Not tested	20	7	12		11	9	
Antimicrobial drug resistance				1.00			0.21
No	84 (98)	54 (98)	27 (96)		38 (95)	46 (100)	
Yes§	2 (2)	1 (2)	1 (4)		2 (5)	0	

*Birthplace information available for 88 persons. RFLP, restriction fragment-length polymorphism.
†Probability of a significant difference between Canada-born and foreign-born persons for each variable; calculated by using the 2-sided Fisher exact test or χ^2 test, as appropriate.
‡Probability of a significant difference between the 2 periods for each variable; calculated by using the 2-sided Fisher exact test or χ^2 test, as appropriate.
§Represents 1 Canada-born person whose isolate was resistant to ethambutol only and 1 foreign-born case whose isolate was resistant to isoniazid only.

Toronto. His clinical and research interests pertain to TB and other infectious diseases affecting immigrant and refugee populations.

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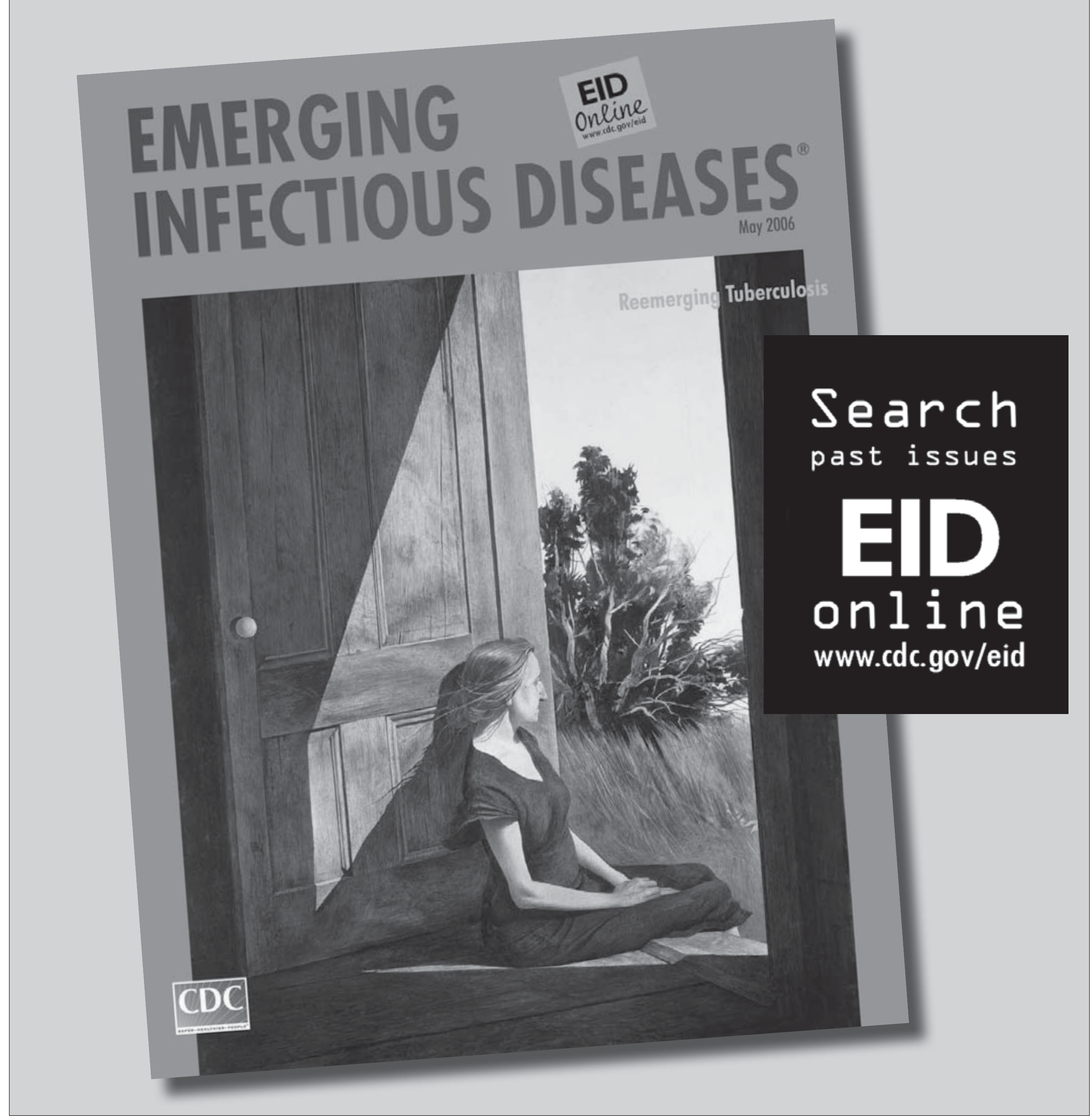

Elephant-to-Human Transmission of Tuberculosis, 2009

Rendi Murphree, Jon V. Warkentin, John R. Dunn, William Schaffner, and Timothy F. Jones

In 2009, the Tennessee Department of Health received reports of 5 tuberculin skin test (TST) conversions among employees of an elephant refuge and isolation of *Mycobacterium tuberculosis* from a resident elephant. To determine the extent of the outbreak and identify risk factors for TST conversion, we conducted a cohort study and onsite assessment. Risk for conversion was increased for elephant caregivers and administrative employees working in the barn housing the *M. tuberculosis*–infected elephant or in offices connected to the barn (risk ratio 20.3, 95% confidence interval 2.8–146.7). Indirect exposure to aerosolized *M. tuberculosis* and delayed or inadequate infection control practices likely contributed to transmission. The following factors are needed to reduce risk for *M. tuberculosis* transmission in the captive elephant industry: increased knowledge about *M. tuberculosis* infection in elephants, improved infection control practices, and specific occupational health programs.

Zoonotic transmission of *Mycobacterium tuberculosis* from elephants to humans working in close proximity was described in the late 1990s. Studies of workers exposed to elephants infected with *M. tuberculosis* have reported a potential for elevated risk among those who have prolonged and close contact with elephants; engage in treatment, medical procedures, or necropsies of elephants; live inside or close to an elephant barn; or participate in cleaning elephant barns or work as groundskeepers (*1–3*).

In North America, ≈270 Asian and ≈220 African elephants live in captivity (*4,5*), most in facilities accredited by the Association of Zoos and Aquariums and the rest in public, private, and nonprofit facilities. Among these, ≈12% of Asian and ≈2% of African elephants are thought to be infected with *M. tuberculosis* (*6,7*). Recommendations for detection and treatment of tuberculosis (TB) in elephants exist (*8*). However, no standard definition exists for latent TB in elephants, and no sound clinical criteria exist for diagnosing TB in elephants. Elephants are considered exposed to *M. tuberculosis* if they have had contact with an *M. tuberculosis* culture–positive animal. They are thought to have active TB when *M. tuberculosis* is cultured from respiratory secretions obtained from their trunk (trunk wash). However, performing a trunk wash is challenging, and culture of *M. tuberculosis* from these specimens is unreliable (*9,10*). Knowledge about effectiveness of human antituberculous medications in elephants is limited (*6–8*).

We describe an outbreak of *M. tuberculosis* infection among employees of an elephant refuge. We also present findings of the ensuing epidemiologic and environmental investigation conducted to identify work practices and facility characteristics that probably contributed to zoonotic transmission.

Outbreak

In July 2009, routine screening detected conversion of tuberculin skin test (TST) results from negative to positive among caregivers at a nonprofit elephant refuge in south-central Tennessee, USA. In addition, records review revealed that respiratory secretions obtained by trunk wash of a quarantined elephant (elephant L) in December 2008 contained *M. tuberculosis*. To determine the extent of the outbreak, identify risk factors for TST conversion among humans, and develop strategies to prevent ongoing zoonotic transmission, we conducted an investigation.

Setting

The elephant refuge was established in 1995 with the mission of caring for sick, old, or abused elephants

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transferred from private owners, zoos, and circuses. It operates on 2,700 acres divided by fences into 3 distinct areas, each having its own barn. Elephants graze outdoors during the day and might be enclosed in barns at night, particularly during cold or inclement weather. At the time of the outbreak, 1 area housed 2 African elephants; 1 area housed 6 Asian elephants; and a third area housed 7 Asian elephants in a large quarantine barn connected to a 2-story administrative support building. The refuge is accredited by the Association of Sanctuaries and licensed by the United States Department of Agriculture (USDA) and the Tennessee Wildlife Resources Agency (TWRA); it is closed to the public.

In 2004, the refuge received, from an exotic animal farm in Illinois, 2 female Asian elephants with a history of active TB. The transfer of *M. tuberculosis* culture–positive elephants into Tennessee was contingent upon adherence to the USDA-endorsed Guidelines for the Control of Tuberculosis in Elephants (*8*) and an infection control plan set forth by TWRA in consultation with the Tennessee Department of Health (TDH). One elephant died of TB in 2005; the other was treated with antituberculous medications for 1 year and was released from isolation in accordance with the guidelines.

In 2006, the refuge accepted 8 additional elephants from the same exotic animal farm in Illinois. Although none were known to have active TB, they were considered exposed and at high risk for latent *M. tuberculosis* infection because they had been housed with *M. tuberculosis* culture–positive elephants. In accordance with the guidelines, all 8 were quarantined when they arrived in Tennessee, and respiratory secretions obtained by trunk wash were tested annually for *M. tuberculosis*. In 2008, one died of causes unrelated to TB.

Investigation

Information and records provided by the refuge and TWRA were used to construct a historical timeline of key events for employees and resident elephants. Onsite evaluations of barn management and husbandry practices were conducted.

Elephants at the refuge had been trained to give respiratory secretions that were used for culture isolation of *M. tuberculosis* by a triple-sample trunk-wash method (*8*). Briefly, 30–60 mL of sterile saline was instilled into the elephant's trunk. The elephant raised and then lowered its trunk to drain or exhale the saline into a plastic bag. Three samples obtained on separate mornings within 1 week were processed by using standard methods for culture isolation of mycobacteria (*11*). *M. tuberculosis* isolates obtained from elephant respiratory secretions were genotyped by using standard methods recommended by the Centers for Disease Control and Prevention (CDC). Results were compared with others stored in the CDC TB Genotyping Information Management System.

A retrospective cohort study was conducted to identify risk factors for *M. tuberculosis* infection among employees who worked at the elephant refuge during 2006–2009. One investigator interviewed current employees in person and former employees by telephone. Employees were asked about potential risk factors for *M. tuberculosis* exposure, history of TSTs and *M. tuberculosis* infection, work assignments and practices, training and use of personal protective equipment, and close contact with elephants. Close contact was defined as touching or being close enough to touch an elephant. TST results, employment history, and N95 respirator fit-testing dates were verified by checking employee records at the refuge.

A preemployment TST was required for elephant caregivers, and all employees received annual TST screening. For employees with a documented negative (<10 mm) TST result, a ≥10 mm increase in induration within 2 years was considered a TST conversion indicative of recent infection with *M. tuberculosis* (*12–14*). Employees whose TST results converted were evaluated for latent *M. tuberculosis* infection or active TB at local health departments or by private clinicians.

Environmental samples were collected from the barn housing the *M. tuberculosis*–infected elephant. These included elephant feces (triplicate samples of 10 g each), water from drinking troughs (triplicate samples of 45 mL each), and swabs of barn surfaces (e.g., duplicate or triplicate samples collected from walls, floors, gates, and drains by using a 3M Sponge-Stick with neutralizing buffer [3M, St. Paul, MN, USA]). Samples were processed by TDH Laboratory Services according to standard methods for culture isolation of mycobacteria (*11*). A theatrical smoke machine was used to enable visualization of air flow patterns within and between the barn and administrative support areas.

Statistical analyses were performed by using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). CDC human subjects review classified this work as public health evaluation and control.

Findings

All trunk-wash specimens obtained from elephants at the refuge during 2006–2009 had negative *M. tuberculosis* culture results except for specimens taken in December 2008 from 1 elephant living in the quarantine area (elephant L). *M. tuberculosis* culture–positive results were received in March 2009. In July 2009, sampling was repeated and culture-positive results were confirmed. Infection control practices were heightened in October 2009. In 2010, treatment of elephant L with antituberculous medication

began but was complicated by the elephant's intolerance to both oral and rectal medical therapy.

M. tuberculosis isolated from elephant L was susceptible to isoniazid, rifampin, ethambutol, pyrazinamide, and streptomycin and had genotype PCR01621. TDH records indicated that genotype PCR01621 had also been isolated from 2 elephants that had died with TB at the refuge in 2005 and 2006. All 3 elephants had spent time at the same exotic animal farm in Illinois, and at least 1 was among the elephants involved in a 1996 outbreak (*2*). *M. tuberculosis* PCR01621 was also isolated from an elephant in Missouri in 2008 and from a human patient who received a diagnosis of active TB in 2004. The human patient lived in California at the time of diagnosis; his potential for exposure to captive elephants was unknown.

Of 57 refuge employees, 46 (81%) were contacted (25 current and 21 former employees). Interviews were conducted with these 30 caregivers, 11 administrators, and 5 maintenance workers. Eleven former employees could not be reached. The average age of respondents was 38 years (range 20–65 years); 31 (67%) were female. All had at least 1 previous negative TST result; 9 had TST results that converted during 2006–2009 (indurations 12–24 mm), although none were identified as having active TB.

Relative risk estimates for traditional risk factors for TST conversion among refuge employees were not statistically significant (Table 1). No human source of *M. tuberculosis* at the refuge was identified. However, employees who worked in the quarantine area during 2009 were significantly more likely than those who did not work there during that period to convert (risk ratio 20.3; 95% confidence interval 2.8–146.7). One employee converted in 2006 after close, prolonged contact with the elephant that died with TB in 2005. The other 8 converted during 2009 and had worked in the quarantine area for ≥4 hours that year. Therefore, we separately examined characteristics of all 13 employees who worked in the quarantine area for ≥4 hours during 2009 and observed their work practices in more detail (Figure).

Among these 13 employees, only 1 who converted had close contact with any elephant (Table 2). Compared with employees who did not convert, fewer employees who converted reported always wearing an N95 respirator when indicated or having the fit of the respirator tested annually.

Also among these 13 employees, 5 were elephant caregivers, 2 were maintenance workers, and 3 were administrators. Caregivers and maintenance workers engaged in aerosol-generating work practices during quarantine barn maintenance. Hay, sawdust, and excrement were swept or shoveled from elephant stalls and yards every day. In addition, the entire barn was cleaned every day with a high-pressure water sprayer. This practice created a dense mist that visibly lingered in the enclosed barn for hours. Although respirators were indicated for persons working inside the quarantine barn, construction of the barn allowed unfiltered air to flow between the barn and the adjacent 2-story administrative support areas where respirators were not worn. These administrative areas included space for barn storage, elephant food preparation, and data entry; a restroom on the first floor; and an office on the second floor. All 3 administrators who worked in these areas had no direct contact with elephants, but their TST results converted.

M. tuberculosis was not isolated from 52 samples collected from the environment of elephant L. *M. fortuitum* complex was identified in fecal samples from 2 elephants, a water sample, and surface swabs of a watering trough and 2 barn drains. Studies using theatrical smoke confirmed that air was shared between the barn and the 2-story administrative support area under normal working conditions.

The refuge infection control plan was intended for implementation when an *M. tuberculosis* culture–positive elephant was in residence. Before arrival of the 2 culture-positive elephants in 2004, employees had received training on the risk for *M. tuberculosis* transmission from elephants to humans, and the refuge established respiratory protection and TST screening programs. By the end of 2005, all elephants living at the refuge were *M. tuberculosis*

Table 1. Exact relative risk for potential risk factors for *Mycobacterium tuberculosis* infection among 46 elephant refuge employees, Tennessee, USA, 2009*

Potential risk factor	TST conversion/ risk factor, no. (%)	TST conversion/ no risk factor, no. (%)	Relative risk (95% CI)†
Foreign born	2/6 (33)	7/40 (18)	1.91 (0.51–7.10)
International travel past 5 y	5/19 (26)	4/27 (14)	1.78 (0.58–5.76)
Exposure to person(s) with TB	0/4 (0)	9/42 (21)	NC
Previous health care facility work	1/8 (13)	8/38 (21)	0.59 (0.09–4.10)
Previous correctional facility work	0/4 (0)	9/42 (21)	NC
Previous homeless shelter work	0/1 (0)	9/45 (20)	NC
Close contact with elephant(s)	2/11 (18)	7/35 (20)	0.91 (0.22–3.75)
Quarantine area exposure during 2009	8/13 (62)	1/33 (3)	20.31 (2.81–146.69)

*TST, tuberculin skin test; CI, confidence interval; TB, tuberculosis; NC, not computed.
†Relative risk and confidence intervals were not computed when at least 1 cell contained zero.

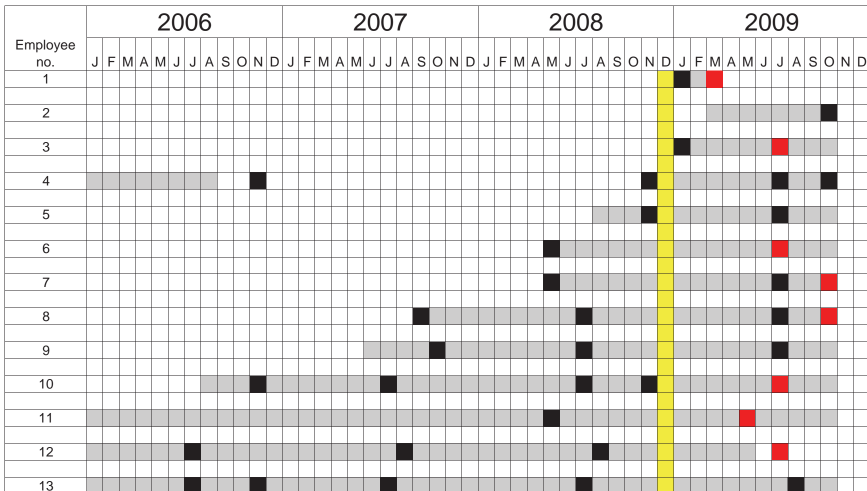

Figure. Tuberculin skin test (TST) conversion timeline for 13 employees who worked in the quarantine area of an elephant refuge, Tennessee, USA, 2009. Gray, exposure to quarantine barn; black, negative TST result; red, positive TST result; yellow, elephant L positive for *Mycobacterium tuberculosis*.

culture negative. Subsequently, formal employee training was discontinued, and infection control procedures were not strictly followed. The refuge continued single preemployment TST screening for elephant caregivers and annual TST screening for all employees.

Discussion

Epidemiologic and observational data indicate that *M. tuberculosis* was transmitted from an elephant with active TB to humans working at the elephant refuge. Employees who worked ≥4 hours in the quarantine barn during 2009 were 20× more likely to have latent *M. tuberculosis* infection than those who did not. TST results for refuge employees without quarantine barn exposure in 2009 did not convert. Risk for employees working in the quarantine area was probably increased by delayed response and failure to enhance infection control practices after obtaining *M. tuberculosis* culture–positive results for elephant L. Notably, close contact with elephant L was not required for transmission. Caregivers and maintenance workers probably aerosolized *M. tuberculosis* that had been expelled or excreted by elephant L while they cleaned soiled barn surfaces. For example, pressure washing created an impressively dense mist that lingered in the barn throughout the day. The mist was not contained within the barn and mixed with air in the connected administrative support areas where respirators were never worn, thus providing a route of indirect *M. tuberculosis* transmission for the 3 administrative workers who reported no contact with elephant L. The hypothesis of indirect transmission is further supported by the TST conversion of a study investigator who spent limited time in the administrative support area before the risk was recognized and interventions were implemented.

During the 19th and 20th centuries, disease caused by *M. tuberculosis* among captive elephants living in Asia, Europe, and North America was sporadically reported (*14*–*19*). The first reported outbreak of TB among elephants in North America occurred at an exotic animal farm in Illinois in 1996 (*2*). The investigation identified evidence of *M. tuberculosis* infection in 4 Asian elephants (3 of which died) and 11 elephant caregivers (1 of whom had active TB). The event prompted action from USDA, and since 1998, the USDA Animal Plant Health Inspection Service has required annual *M. tuberculosis* testing by the trunk-wash–culture method for all captive elephants in the United States (*20*).

Analogous to culture-positive sputum in human patients, an *M. tuberculosis* culture-positive trunk wash from an elephant is considered the standard for confirming active TB disease. However, active TB disease and shedding of *M. tuberculosis* organisms cannot be excluded

Table 2. Exact relative risk for potential risk factors for latent *Mycobacterium tuberculosis* infection among 13 employees who worked in the quarantine area of an elephant refuge, Tennessee, USA, 2009*

Potential risk factor	TST conversion/ risk factor, no. (%)	TST conversion/ no risk factor, no. (%)	Relative risk (95% CI)†
Close contact with elephant(s)	1/3 (33)	7/10 (70)	0.48 (0.09–2.48)
Participated in elephant trunk washes	0/1 (0)	8/12 (67)	NC
Pressure washed barn walls and floors	5/8 (63)	3/5 (60)	1.04 (0.43–2.55)
N95 respirator fit tested annually	2/5 (40)	6/8 (75)	0.53 (0.17–1.68)
“Always” compliant with N95 wear	2/5 (40)	6/8 (75)	0.53 (0.17–1.68)

*TST, tuberculin skin test; CI, confidence interval; NC, not computed.
†Relative risk and confidence intervals were not computed when at least 1 cell contained zero.

with a culture-negative trunk-wash result because the test has low sensitivity (*9,10,21*).

Considerable effort has gone toward developing methods for early and reliable diagnosis of latent *M. tuberculosis* infection among elephants. TST is unreliable (*10*), but serum antibody tests appear promising (*9,22*). Although serologic tests can detect infection with *M. tuberculosis* years earlier than trunk-wash cultures (*9*), negative serologic results cannot exclude the possibility of infection. In February 2010, USDA added serologic testing (ElephantTB STAT PAK Kit; Chembio Diagnostic Systems, Inc., Medford, NY, USA) to its annual trunk-wash culture requirement for all elephants (*8*).

Knowledge gaps exist about the timing between elephant exposure, seroconversion, latent infection, active disease, and shedding. To improve medical management of elephants and to reduce the risk for transmission to other animals and humans, a better understanding of *M. tuberculosis* infection among elephants is crucial. Gaps also exist in knowledge regarding treatment and cure of elephants with *M. tuberculosis* infection. Although antituberculous medications used to treat humans are thought to be effective for treating active TB in elephants, little evidence is available to guide decisions regarding medication choice, dosage, length of treatment, or assessment for cure. Also unclear is whether treatment of elephants with *M. tuberculosis* infection successfully prevents progression to active TB disease.

Our findings highlight the effects of gaps in scientific knowledge and provide new information on potential risk factors for zoonotic transmission of *M. tuberculosis*. First, in this outbreak the inability to accurately and expeditiously detect *M. tuberculosis* infection and disease in elephants contributed to unrecognized, and therefore uncontrolled, risk. Improved methods for diagnosis of *M. tuberculosis* infection in elephants are needed. Second, infection control practices were insufficient to protect employees, creating an argument for detailed evidence-based guidelines and a more comprehensive approach to implementation. Third, employees were largely unaware of the risk for zoonotic *M. tuberculosis* transmission and the need to use adequate respiratory protection. Because risk cannot be eliminated, a strong occupational health and training program is needed for employees who work in facilities that house elephants potentially exposed to *M. tuberculosis*. Finally, our study suggests that employees without close contact with elephant L were infected through indirect transmission of *M. tuberculosis* aerosolized during routine barn maintenance (i.e., pressure washing or sweeping waste) or suspended in shared air. Reasonable efforts to reduce aerosol-generating practices and to limit aerosol spread in this unique environment should be considered.

The One Health movement argues for integrating human and veterinary medicine to defend the health and well-being of all animal species (*23*). This report provides a textbook illustration of this need. Captive elephants have emerged as an unanticipated source of *M. tuberculosis* infection among humans and therefore must be integrated in our strategies to control and eliminate TB. Because of the gaps in scientific knowledge, the high prevalence of *M. tuberculosis* infection among elephants living in North America, and the insensitivity of diagnostic tests, a substantial need exists for focusing attention on infection control practices and occupational health programs specifically designed to reduce zoonotic *M. tuberculosis* transmission in the captive elephant industry.

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Dr Murphree is a CDC Epidemic Intelligence Service Officer interested in the epidemiology of emerging and zoonotic infectious diseases. She dedicates this article to the memory of her grandfather, Ralph Cleo Cowart.

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Mycobacterium tuberculosis Cluster with Developing Drug Resistance, New York, New York, USA, 2003–2009

Bianca R. Perri, Douglas Proops, Patrick K. Moonan, Sonal S. Munsiff, Barry N. Kreiswirth, Natalia Kurepina, Christopher Goranson, and Shama D. Ahuja

In 2004, identification of patients infected with the same *Mycobacterium tuberculosis* strain in New York, New York, USA, resulted in an outbreak investigation. The investigation involved data collection and analysis, establishing links between patients, and forming transmission hypotheses. Fifty-four geographically clustered cases were identified during 2003–2009. Initially, the *M. tuberculosis* strain was drug susceptible. However, in 2006, isoniazid resistance emerged, resulting in isoniazid-resistant *M. tuberculosis* among 17 (31%) patients. Compared with patients with drug-susceptible *M. tuberculosis*, a greater proportion of patients with isoniazid-resistant *M. tuberculosis* were US born and had a history of illegal drug use. No patients named one another as contacts. We used patient photographs to identify links between patients. Three links were associated with drug use among patients infected with isoniazid-resistant *M. tuberculosis*. The photographic method would have been more successful if used earlier in the investigation. Name-based contact investigation might not identify all contacts, particularly when illegal drug use is involved.

Name-based contact investigation is a core tuberculosis (TB) control method, yet its limitations are documented (*1–9*). Although name-based contact investigations can elucidate TB transmission chains, these investigations are typically limited to household and other close contacts (*10,11*). Molecular characterization of *Mycobacterium tuberculosis* (i.e., TB genotyping), when combined with contact investigation, can increase screening yield and identify transmission venues, particularly among populations at high risk (e.g., substance users, immigrants, and other hard-to-reach populations) (*2–5, 12*,*13*).

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Since 2001, the New York City (NYC) Bureau of Tuberculosis Control (BTBC), New York, New York, USA, has conducted universal genotyping and used results to detect and investigate clusters of TB with suspected recent transmission (*14*). One cluster, first identified and characterized in NYC in 2004, was the focus of an extensive epidemiologic investigation. We describe the investigation and discuss novel methods used during the investigation to understand TB transmission.

Materials and Methods

Since January 1, 2001, all initial culture-positive *M. tuberculosis* isolates have been characterized by using spacer oligonucleotide type analysis (spoligotyping) at the New York State Department of Health Wadsworth Center (Albany, NY, USA) and IS*6110* restriction fragment length polymorphism (RFLP) typing at the Public Health Research Institute Tuberculosis Center (Newark, NJ, USA) (*14*). In accordance with Centers for Disease Control and Prevention (CDC) National Tuberculosis Genotyping Service, isolates were sent to the Michigan Bureau of Laboratories (Lansing, MI, USA) for 12-loci mycobacterial interspersed repetitive-unit variable-number tandem repeat (MIRU-VNTR) analysis (*15*).

Case Definition

Cluster membership was defined as patients who had a diagnosis of TB in NYC during 2003–2009 and whose isolates had identical spoligotype and IS*6110* RFLP

patterns. As the investigation continued, this definition was expanded and included patients whose isolates had identical spoligotype, 12-loci MIRU-VNTR results, and IS*6110* RFLP patterns with ± 1 band.

Drug Susceptibility Testing

TB drug susceptibility testing (DST) was performed at the NYC Public Health Laboratory and the Wadsworth Center on initial *M. tuberculosis* isolates by using either BACTEC 460 or Mycobacterial Growth Indicator Tube 960 (Becton Dickinson, Sparks, MD, USA). A standard agar-proportion method with Middlebrook 7H10 media was used to confirm resistance (*16–18*). If DST indicated isoniazid resistance, DNA sequencing of the catalase–peroxidase G (*katG*) and enoyl reductase A (*inhA*) genes (*19*) was performed at the Public Health Research Institute Tuberculosis Center.

Contact Investigation

Contact investigations were conducted per BTBC guidelines (*20*). For contacts of infectious index patients, staff assessed hours of TB exposure during infectious periods of patients, defined as the 12-week period before the patient began appropriate TB treatment (*20*,*21*). Infectious periods were extended to date of symptom onset if TB symptoms started >12 weeks before treatment began. Contacts having documented latent TB infection or TB symptoms were referred for medical evaluation and treatment.

Cluster Investigation

An investigation of patients with the same *M. tuberculosis* strain was initiated to identify chains of transmission within the cluster and uncover epidemiologic links between TB patients. An epidemiologic link between 2 patients indicated that patients were linked by person, place, or time. Definite epidemiologic links between patients required 1 of the following criteria: named another patient as a contact, had a common contact, reported being in the same location during a patient's infectious period, or recognized each other's names or photographs. Probable epidemiologic links indicated that patients were in the same location during the same date range regardless of the infectious period of either patient or that 1 patient recognized another's name or photograph. Possible epidemiologic links occurred when patients lived or visited an area within 0.8 km (0.5 miles) of another or had a similar social environment. If >1 link was established between 2 patients, the strongest link was counted.

Routine demographic and clinical data were obtained from the NYC TB registry and patient interviews. Additional data on homelessness and correctional history were obtained from NYC and New York State databases. Information regarding contacts and places of association (e.g., residences, worksites, and schools) of patients was analyzed to establish links between patients and to derive transmission hypotheses. To substantiate these hypotheses, we reinterviewed patients and their contacts by using a structured questionnaire. The questionnaire was updated with information obtained during patient interviews to ensure that hypotheses were reassessed throughout the investigation.

In October 2007, the NYC Department of Health and Mental Hygiene (DOHMH) Office of General Counsel approved use of names and photographs of patients and their contacts during interviews by BTBC. Cluster investigators administered informed consent forms. Consent forms indicated that names or photographs would be obtained and shown to persons being interviewed as part of the cluster investigation. If the patient denied voluntary permission but had an incarceration history, a public record booking photograph was used. To avoid disclosing confidential medical information, fictitious names and unrelated photographs were included in the compilation of names and photographs. Investigators did not confirm or deny a TB diagnosis of any person or how persons were related. During interviews, investigators asked if patients or contacts recognized any names or photographs. If recognition was indicated, the interviewer probed to understand how persons were linked.

Statistical Analysis

We compared categorical data by using Pearson χ^2 or Fisher exact tests, as appropriate. For continuous data, the Mann-Whitney test was used to compare medians. Statistical analyses were conducted by using SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA).

Places of association were geocoded through the NYC Department of City Planning's Geosupport Desktop Edition Software 9.6.9. Geocoded locations were imported into ArcGIS 9.2 (ESRI, Redlands, CA, USA) and mapped. Locations not geocoded by street address were geocoded by street intersection or other features. The ArcGIS point distance geoprocessing tool was used to calculate Euclidean distances between places of association of patients. Data were obtained as part of an outbreak investigation. Therefore, NYC DOHMH and CDC deemed this activity nonhuman subjects research.

Results

During 2003–2009, we identified 54 cases of TB as part of this cluster (Figure 1). Patient residence at TB diagnosis by NYC neighborhood is shown in Figure 2. Among 35 (65%) patients who lived in Upper Manhattan at diagnosis, median distance between the residence of any 2 patients was 1.4 km (range 0.01 km–6.6 km). Median

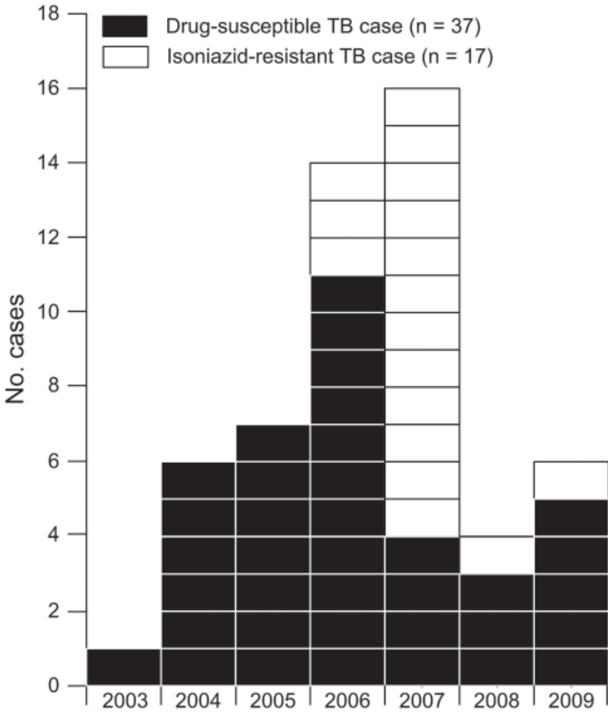

Figure 1. Cluster of 54 cases of tuberculosis (TB), by year of diagnosis, New York, New York, USA, 2003–2009. The 54 cases include 1 in a patient in the city of New York who was given a diagnosis of drug-susceptible *Mycobacterium tuberculosis* infection in 2007 that was counted by New York State.

distance between any 2 patients residing in the South Bronx (n = 10) at diagnosis was 2.9 km (range 0 km–5.8 km). Initially, the strain was susceptible to first-line anti-TB drugs. However, in 2006, isoniazid resistance emerged in a patient isolate at TB diagnosis. By 2009, 17 (31%) patients had isoniazid-resistant *M. tuberculosis* at diagnosis. All isoniazid-resistant isolates had the Ser315Thr mutation in the *katG* gene and no mutations in the *inhA* gene region sequenced.

TB Genotyping

Forty-seven (87%) of the 54 patients had isolates with a matching spoligotype, IS*6110* RFLP pattern, and 12-loci MIRU-VNTR result (Figure 3). Forty-eight (89%) isolates met the original cluster case definition. Six (11%) were identified as cluster-associated patients on the basis of the expanded cluster case definition. As of December 31, 2008, within the CDC National Tuberculosis Genotyping Service database of 32,581 patient isolates, 6 with this cluster's spoligotype and 12-loci MIRU-VNTR result were reported outside NYC (New York [n = 3], Delaware [n = 1], Georgia [n = 1], and Pennsylvania [n = 1]) (*22*). Among the 3 patients who resided in New York State, 1 was diagnosed in NYC and is therefore counted in the cluster (Figure 1); no link to NYC was identified for the other 2 patients.

Patient Characteristics

Patient median age was 41 years (range 10–77 years); 74% were non-Hispanic black and 69% were male (Table). Among 37 patients with drug-susceptible *M. tuberculosis*, 73% were male and 38% were foreign born. The 17 patients with isoniazid-resistant *M. tuberculosis* were predominately US born (82%) and had a history of illegal drug use (59%) or incarceration (47%).

The shift of patient characteristics with time is shown in Figure 4. During 2003–2005, before isoniazid resistance emerged, 9 (64%) of 14 patients were US born and 4 (44%) of the US-born patients reported illegal drug use. Three patients, of whom 2 attended the same mosque, had a country of origin in West Africa; none reported drug use. In 2006, the number of patients with drug-susceptible *M. tuberculosis* peaked at 11, of whom 8 (73%) were foreign-born. All 4 patients from West Africa with drug-susceptible *M. tuberculosis* had a history of attending different mosques, and 2 had a history of illegal drug use. In 2007, when patients with isoniazid-resistant *M. tuberculosis* were more numerous than those with drug-susceptible *M. tuberculosis*, all 16 patients were US born; 8 (50%) were associated with illegal drug use. Of these 8 patients, 7 (88%) had isoniazid-resistant *M. tuberculosis*.

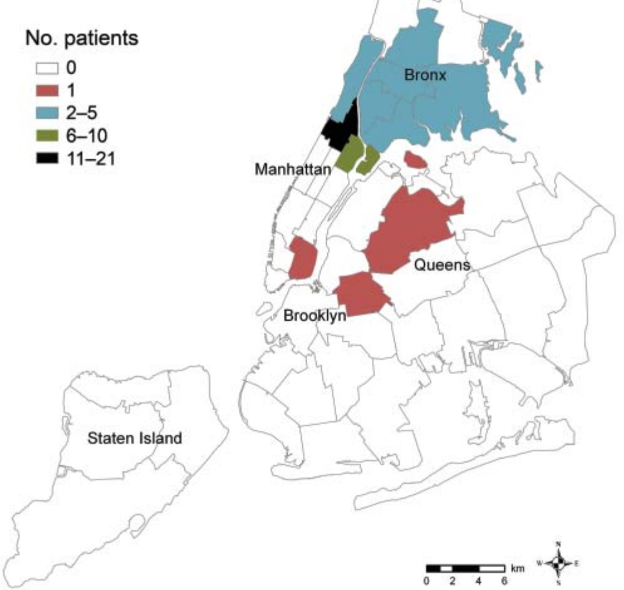

Figure 2. Residences of patients (n = 54) at time of tuberculosis (TB) diagnosis, by neighborhood, New York, New York, USA, 2003–2009. Forty-two neighborhoods were designated by the United Hospital Fund. Each neighborhood is defined by several adjoining ZIP codes (www.nyc.gov/html/doh/html/epi/mapgallery.shtml).

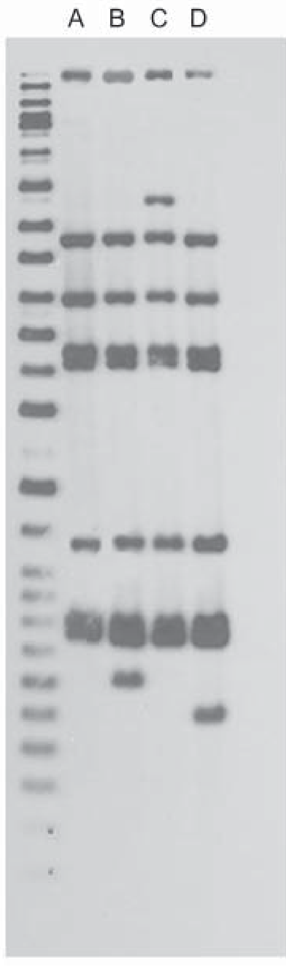

Figure 3. IS*6110* restriction fragment length polymorphism patterns for tuberculosis patients, New York, New York, USA, 2003–2009. Left lane, molecular mass ladder; lane A, n = 48; lane B, n = 1; lane C, n = 1; lane D, n = 4. Spoligotype results (octal code designation) were 777777774020771 for 54 patients. Twelve-loci mycobacterial interspersed repetitive-unit variable-number tandem repeat results were 225313153321 for 53 patients and 2253131–3321 for 1 patient; the dash indicates that there was no peak at this locus for this 1 patient, and the patient with this isolate met the original cluster case definition.

Among the 22 persons who disclosed a history of illegal drug use, 19 (86%) indicated noninjection drug use. The mother of a child with isoniazid-resistant *M. tuberculosis* also reported using illegal drugs. Drugs mentioned by patients connected to illegal drug use included smoking or snorting cocaine (n = 15), smoking marijuana (n = 6), and using heroin (n = 3).

Contact Investigation

Among 48 patients eligible for contact investigation, 1,226 contacts were identified (median 9, range 0–153 contacts/patient). Twelve investigations of TB exposures in congregate settings were conducted. None of the clustered patients named one another as contacts. Contact investigation identified 1 clinically diagnosed TB case linked to a cluster-associated patient with drug-susceptible *M. tuberculosis*.

Cluster Investigation

All 2-patient combinations (n = 1,431) were analyzed for epidemiologic links. Routine cluster investigation identified 3 definite epidemiologic links; only 1 of these links involved a patient with isoniazid-resistant *M. tuberculosis*. One definite epidemiologic link was based on a common contact between a patient with drug-susceptible *M. tuberculosis* and a patient with isoniazid-resistant *M. tuberculosis*. The other 2 links were based on patients living in the same apartment building during the infectious period of 1 of the patients. Cluster investigation methods identified 3 probable epidemiologic links; all involved patients attending the same mosque during an overlapping date range. All 54 patients had a possible epidemiologic link to at least 1 other cluster patient; 98% of patients had multiple possible epidemiologic links. Of the possible epidemiologic links identified, 81% were geographic and 29% involved illegal drug use. Other possible epidemiologic links were identified on the basis of shared patient characteristics such as having a country of origin in West Africa, being infected with HIV, and history of mosque attendance, taxi driver occupation, incarceration, or homelessness.

During 2007–2009, patients were asked for permission to use names and photographs. Ten (59%) of the 17 patients with isoniazid-resistant *M. tuberculosis*, 1 (17%) of 6 patients with drug-susceptible *M. tuberculosis*, and 7 (64%) of 11 contacts granted permission. Public-record booking photographs were used for 2 patients. Four additional probable epidemiologic links were established through name and photograph use; all were associated with illegal drug use. Patients did not indicate familiarity with fictitious names and unrelated photographs that were presented.

Discussion

Despite using substantial resources within BTBC and beyond, we did not clearly identify chains of transmission in this outbreak. Only 3 definite epidemiologic links were identified between patients, and only 1 was associated with the rapidly emerging or spreading isoniazid-resistant strain. The strongest link of this cluster is geographic; patients primarily spent time in the same neighborhoods. Although matching genotype does not always signify recent transmission, geospatial concentration and epidemiologic data indicate ongoing and recent transmission of this rare genotype in NYC. Contact investigation results showed evidence of possible transmission. However, no confirmed

Table. Characteristics of 54 TB patients, by drug susceptibility test results, New York, New York, USA, 2003–2009*

Characteristic	All patients	Type of *Mycobacterium tuberculosis*		p value
		Drug-susceptible, n = 37	Isoniazid-resistant, n = 17	
Median age at TB diagnosis, y (range)	41 (10–77)	42 (12–77)	39 (10–52)	0.12
Male sex	37 (69)	27 (73)	10 (59)	0.30
Race/ethnicity				
Asian	1 (2)	1 (3)	0	1.00†
Hispanic	13 (24)	8 (22)	5 (29)	0.73†
Black, non-Hispanic	40 (74)	28 (76)	12 (71)	0.74†
Country of origin				
United States	36 (67)	22 (59)	14 (82)	0.10
Foreign	17 (31)	14 (38)	3 (18)	0.14
Unknown	1 (2)	1 (3)	0	1.00†
History of illegal drug use‡	22 (41)	12 (32)	10 (59)	0.07
History of homelessness	13 (24)	8 (22)	5 (29)	0.73†
History of incarceration	12 (22)	4 (11)	8 (47)	<0.01†
Pulmonary site of TB§	48 (89)	32 (87)	16 (94)	0.65†
Cavitary (among cases with pulmonary site of disease)	12 (25)	7 (22)	5 (31)	0.50†
Acid-fast bacilli smear positive for respiratory specimen	38 (70)	24 (65)	14 (82)	0.19
HIV status				
Positive	14 (26)	9 (24)	5 (29)	0.74†
Negative	37 (69)	26 (70)	11 (65)	0.68
Unknown	3 (6)	2 (5)	1 (6)	1.00†

*Values are no. (%) unless otherwise indicated. TB, tuberculosis.
†By Fisher exact test.
‡Use of injection (e.g., heroin) or noninjection (e.g., marijuana or cocaine) drugs indicated on any patient record.
§Includes patients with only pulmonary sites of disease and patients with pulmonary and extrapulmonary sites of disease.

secondary TB cases were identified among >1,200 identified contacts, further demonstrating limitations of name-based contact investigation.

This outbreak was only identified through genotyping. PCR-based methods (spoligotyping and 12-loci MIRU-VNTR analysis) better defined this TB cluster. Supplementing contact investigation with laboratory tools to examine strain relatedness (e.g., real-time genotyping and DST) can help TB control program staff identify and investigate outbreaks. Although all patient specimens had a matching genotype, DST results showed 2 phenotypes, and therefore ≥2 distinct transmission chains within the cluster. Identifying separate transmission chains enabled cluster investigators to develop and test hypotheses specific to each chain of transmission. Common characteristics within each transmission chain implied discrete social networks, but these networks could not be confirmed by using routine cluster investigation methods.

Emergence of isoniazid resistance in this cluster cannot be clearly explained. None of the patients with drug-susceptible *M. tuberculosis* showed failure of treatment. Presumably, 1 person, identified by investigators as a shared contact between a patient with drug-susceptible *M. tuberculosis* and a patient with isoniazid-resistant *M. tuberculosis*, had a history of taking medications for TB and showed development of isoniazid-resistant *M. tuberculosis* that had not been reported to BTBC. This person died; therefore, cluster investigators were unable to confirm this hypothesis despite medical record review and pharmacy surveillance.

This investigation was limited by patients' unwillingness to report their contacts, possibly because of fear of disclosing immigration status (not asked by BTBC staff), illegal drug use, or involvement in other illicit activities. Other possible explanations include forgetting or not knowing their contacts by name (*2*,*23*). Certain patients used aliases (not tracked in the NYC TB registry) and claimed to only know their contacts by first names or aliases. Pervasiveness of aliases within patient social networks stymied contact investigation efforts and made establishing epidemiologic links between patients difficult.

High prevalence of illegal drug use within the cluster led investigators to explore how specific drug-use practices contribute to TB transmission. Studies reported that such specific drug-use practices as shotgunning (inhaling smoke from rock cocaine or marijuana and blowing the smoke directly into the mouth of another) and hotboxing (smoking drugs in a small, enclosed space to maximize narcotic effect through first-hand and second-hand smoke) were associated with TB transmission (*24*,*25*). Although these practices were not specifically mentioned by patients or their contacts, specific questions were not asked until later in the investigation. After consulting with substance-use experts, BTBC revised their cluster-investigation questionnaire and provided investigators with additional training on patient-interview procedures and drug-use

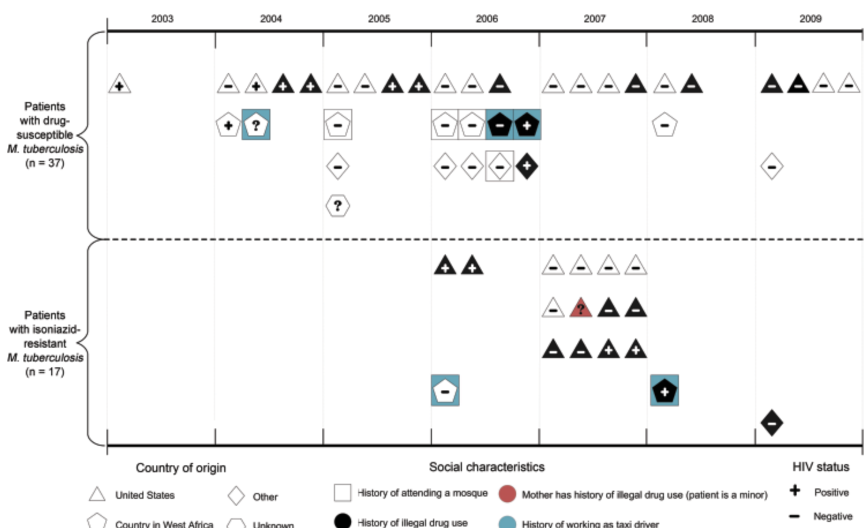

Figure 4. Common characteristics among 54 patients infected with *Mycobacterium tuberculosis*, by year of tuberculosis diagnosis and drug susceptibility testing results, New York, New York, USA, 2003–2009.

subculture. Understanding drug-use behavior helps TB control personnel elicit sensitive transmission information. BTBC also modified how substance-use information is collected and recorded in the TB registry.

Transmission through casual contact and increased virulence are possible explanations for extensive transmission of this strain and lack of recognition among patients. Although TB transmission from casual contact is considered rare, it has been documented (*26*–*30*). If this strain, like other outbreak strains (*29*), was highly virulent, extensive transmission among patients who did not recognize each other would have been possible. Moreover, geographic proximity of patients to one another might have increased opportunities for TB exposure and supported transmission through casual contact. In addition, positive results for acid-fast bacilli in smears of respiratory specimens among cluster-associated patients were substantial (70% overall, 93% among cocaine users) and considerably greater than recent past NYC TB patients (range 42%–46% during 2003–2008) (NYC DOHMH, unpub. data), thus increasing likelihood of transmission. Investigation findings were consistent with those of a London study that reported that pulmonary TB patients who used cocaine were more likely to be sputum smear positive at diagnosis (*31*), perhaps related to delays in seeking medical care.

Photograph and name use yielded the strongest epidemiologic links between patients with isoniazid-resistant *M. tuberculosis*. It was the only method that confirmed patient recognition within the cluster. All epidemiologic links established through photograph recognition were related to illegal drug activity. Other outbreak investigations have highlighted unwillingness of patients to share social contacts when these contacts are connected to illegal activities (*4*,*5*,*13*).

Insights gained from using name and photograph data in an ongoing investigation will benefit TB control programs. This method would have been more successful if used earlier in the investigation. TB control personnel contemplating adopting this strategy should obtain legal guidance before an outbreak occurs because privacy laws vary from one locality to another.

This outbreak investigation highlights an array of challenges for US-based TB control programs. Understanding and preventing TB transmission among hard-to-reach populations requires considerable resources. Conventional contact investigation can be inadequate for identifying and curtailing TB transmission among difficult-to-reach- populations. New methods, including using name and photograph data, are needed for TB elimination.

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Reduction of *Coxiella burnetii* Prevalence by Vaccination of Goats and Sheep, the Netherlands

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Recently, the number of human Q fever cases in the Netherlands increased dramatically. In response to this increase, dairy goats and dairy sheep were vaccinated against *Coxiella burnetii*. All pregnant dairy goats and dairy sheep in herds positive for Q fever were culled. We identified the effect of vaccination on bacterial shedding by small ruminants. On the day of culling, samples of uterine fluid, vaginal mucus, and milk were obtained from 957 pregnant animals in 13 herds. Prevalence and bacterial load were reduced in vaccinated animals compared with unvaccinated animals. These effects were most pronounced in animals during their first pregnancy. Results indicate that vaccination may reduce bacterial load in the environment and human exposure to *C. burnetii.*

Q fever, which is caused by *Coxiella burnetii*, is a worldwide zoonotic infectious disease, and ruminants are the main reservoir for human infections (*1–3*). Ruminant infections may occasionally result in abortions, which are associated with shedding of large amounts of bacteria in placentas and birth fluids (*4*). Human infections have been reported mainly in persons handling infected animals and their products (*5–8*). However, this disease has not been perceived as a major public health risk for the general population. In 2007, a major epidemic occurred in the general population in the Netherlands (*9*), which resulted in >2,300 reported cases in 2009. An explanation for the emergence of human Q fever was abortion clusters in goat herds beginning in 2005 within an intensified dairy goat production system (*10–15*). This hypothesis was substantiated by epidemiologic studies, which indicated a possible spatial link between dairy goat farms and human cases (*16*).

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Reduction of the number of human cases was considered essential by public health authorities in the Netherlands. One of the intervention measures taken was vaccination of dairy goats against *C. burnetii* (*17*). This measure assumed that vaccination would reduce abortions and bacterial shedding to levels that would reduce the number of human cases in the following year. Vaccination began in 2008 and intensified in 2009. As the number of cases of *C. burnetii* infection in patients doubled in 2009, policymakers applied a precautionary principle and decided to cull all pregnant dairy goats or sheep on infected farms before the 2010 kidding season. This measure was implemented at the end of 2009 and thereby precluded any field analysis of vaccine efficacy in the spring of 2010. However, there was an opportunity to sample animals shortly after they were humanely killed. The purpose of this study was to quantify the effect of vaccination on bacterial load in excreta of pregnant animals.

Materials and Methods

Q Fever in the Netherlands since 2005

Human Q fever cases in the Netherlands increased from 168 in 2007 to 1,000 in 2008 and 2,355 in 2009, mainly in Noord-Brabant Province (*11*). A campaign of voluntary vaccination of dairy goats began at the end of 2008 in the area of the 2007 human case cluster and was followed by mandatory vaccination of all dairy goat and dairy sheep on farms with >50 animals in a larger area in 2009. This vaccination zone included Noord-Brabant Province and parts of adjacent provinces because the

supply of vaccine was not sufficient for all small ruminant farms in the Netherlands and because most human cases had occurred in that area (Figure 1) (*13*).

Additional control measures implemented in the fall of 2009 were a bulk milk test every 2 weeks to detect *C. burnetii*–infected herds and to monitor *C. burnetii*–negative herds, movement and breeding bans for dairy goats or sheep, and culling of all pregnant dairy goats or sheep on infected farms. Health authorities considered a farm to be infected when 2 consecutive bulk milk samples were positive by PCR, as tested by 2 laboratories, including the national reference laboratory (*17*). Thus, culling included pregnant goats in vaccinated herds and pregnant goats in unvaccinated herds located outside the vaccination zone. Culling was conducted from the end of December 2009 through May 2010.

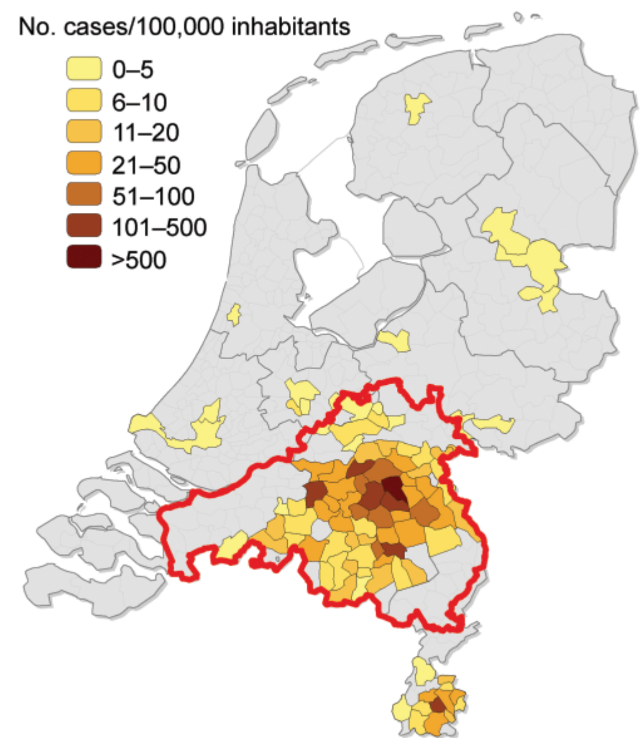

Figure 1. Density of 1,133 reported cases of acute Q fever in humans per municipality, the Netherlands, January 1–June 10, 2009. Area outlined in red is where vaccination of dairy goats and sheep was mandatory in 2009 (Noord Brabant Province and parts of adjacent provinces). Data were obtained from the National Institute for Public Health and the Environment, Statistics Netherlands, the Food and Consumer Product Safety Authority, and the Ministry of Agriculture, Nature and Food Quality.

Vaccine

The vaccine used was Coxevac (Ceva Santé Animale, Libourne, France). This vaccine was not registered in the Netherlands at the time of the study, but authorities had issued a temporary exemption. The vaccine is a phase I vaccine containing inactivated *C. burnetii* strain Nine Mile (*18*). It was recommended that uninfected animals be vaccinated twice over a 1-month interval before pregnancy. Although efficacy in dairy goats was not shown, the expected effects in vaccinated animals were reduced infection, abortion, and bacterial shedding if animals were infected after vaccination (*19*–*21*).

Study Design

For various reasons related to regulations of the national culling operation, unvaccinated dairy goats from 5 farms, vaccinated dairy goats from 7 farms, and unvaccinated dairy sheep from 1 farm were included in this study. Farms were not randomly selected but were selected on the basis of convenience of culling date, vaccination status, and agreement of farmers to participate in the study. We sampled 100 animals per farm, 50 pregnant and lactating animals (old animals), and 50 nulliparous animals (young animals). With this sample size, we expected to be able to detect a 20% difference in *C. burnetii* prevalence between vaccinated and unvaccinated animals and between old and young animals. We tested 3 types of samples: 1) uterine fluid, to detect animals with a high risk for shedding around parturition; 2) vaginal mucus, to be consistent with test results of other studies (*19*–*21*); and 3) milk, because herds were monitored on the basis of results of bulk milk tests.

On the day before culling, animals were selected and marked on the farm by the study team; authorities identified pregnancies by using sonography. We selected pregnant animals that were closest to giving birth because it was expected that these animals had the highest number of *C. burnetii* in birth fluids, which would facilitate detection of infection (*4*). After animals were humanely killed on farms, marked animals were transported in a separate container to a rendering plant (Rendac BV, Son, the Netherlands), where they were unloaded onto a concrete floor and prepared for sampling.

Sampling

Uterine fluid was obtained by using a 9-mL monovette EDTA blood collection system (Sarstedt, Nümbrecht, Germany) and a Bovivet 2.10 mm × 60 mm needle (Terumo Europe NV, Leuven, Belgium). Before obtaining uterine fluid, we made an incision in the linea alba cranial from the udder, moved part of the uterus to an extraabdominal position, and cleaned the uterus with alcohol-soaked cotton balls. We also cleaned the vulva with alcohol-soaked cotton balls and then obtained a swab sample from the vagina wall by using a dry and sterile cotton-tipped Cultiplast swab (LP Italiana SPA, Milan, Italy). These 2 samples were obtained from all selected animals. Additionally, from old animals

we obtained a milk sample, which was collected into a 30-mL sterile tube. The teat was cleaned with alcohol-soaked cotton balls before sampling, and the first few streams of milk were discarded. All samples were frozen at –40°C within a few hours after sampling and were sent to the laboratory to be analyzed after the end of the culling period.

Diagnostic Test

Quantitative real-time PCR was performed for all samples. Milk samples were analyzed at the Animal Health Service by using the Taqvet *Coxiella burnetii* TaqMan Quantitative PCR (Laboratoire Service International, Lissieu, France). Swabs and uterine samples were analyzed by the national reference laboratory by using an in-house real-time PCR specific for the *C. burnetii* insertion sequence 1111a gene (*22*). Results for the 3 sample types were given as positive, negative, or doubtful on the basis of cycle threshold (C_t) values, in which a value <36.01 was considered positive and a value >40 was considered negative. A negative result indicated that no specific signal was detected in a maximum of 40 cycles. Values between 36.01 and 40 were reported as doubtful on the basis of <100% reproducibility. For additional analysis, we considered all samples with $C_t \leq 40$ as positive.

Statistical Analyses

Vaccine efficacy was calculated for young and old animals separately for all 3 sample types according to the following equation: [% (positive test result, unvaccinated) – % (positive test result, vaccinated)] / [% (positive test result, unvaccinated)] (*23*). This efficacy can be interpreted as the percentage of positive samples ($C_t \leq 40$) prevented by vaccination in a vaccinated population.

Influence of vaccination and parity on test results of individual animals was examined by using logistic regression (*24*) for the 3 sample types. We included vaccination status and parity group in the model as explanatory variables. Herd was included as a random factor to incorporate the fact that observations within a herd are dependent in the model. For uterine samples and vaginal swabs, we used the equation logit (fraction of positive test results) = parity (old) + vaccination status stratified by parity (young or old vaccinated) + random herd effect stratified by vaccination status (vaccinated or unvaccinated herds). For milk samples, we used the equation logit (fraction of positive test results) = vaccination status (vaccinated) + random herd effect stratified by vaccination. Vaccine effect was quantified by calculating the odds ratio (OR).

For positive samples only, we tested whether vaccination had an effect on the relative amount of bacteria present in each sample type, as indicated by the C_t value. A C_t value closer to 0 indicates a higher bacterial concentration in the sample relative to a C_t value closer to 40. We performed survival analysis on samples with C_t values for which the C_t value at which a sample result becomes positive is considered the event. Hazard ratio (HR) indicates the rate at which samples from unvaccinated animals become positive compared with samples from vaccinated animals (*25*). No correction for herd level was necessary and no correction for parity was possible because of the low number of bacterial shedders per group. For each of the 3 samples types, we used the equation C_t value (of positive samples only) = vaccination status (vaccinated).

Kaplan-Meier curves were plotted to show bacterial load in samples from old vaccinated, young vaccinated, old unvaccinated, and young unvaccinated animals. Statistical analyses were performed by using R software (*26*). For logistic regression, the function glmer() in lme4 in R software (*27*) was used. For survival analysis, the functions Surv() and coxph() in Survival in R software (*28*) were used. The model fit of all models was assessed by using the likelihood ratio test.

Results

Background Information for Individual Farms

Information for each farm is shown in Table 1. Three farms (B, F, and K) did not have a history of animals with Q fever before the end of 2009 when their bulk milk PCR results changed from negative to positive during the monitoring period, which suggested a recent infection. Abortion caused by Q fever had been diagnosed in 2008 on sheep farm X. On all other farms, ≥1 bulk milk ELISA or PCR results were positive for *C. burnetii* in 2008 or 2009. Animals in vaccinated herds were supposedly vaccinated twice in 2009, with the exception of farm M, where the first vaccination was given after abortions had occurred.

Effect of Vaccination on Bacterial Shedding

Crude test results are summarized in Table 2. The percentage of *C. burnetii*–positive animals on each farm is shown in Figure 2. For vaccinated animals, 0.43% of uterine samples, 30% of vaginal swabs, and 4% of milk samples were positive (C_t <36.01). For unvaccinated animals, 26% of uterine samples, 76% of vaginal swabs, and 33% of milk samples were positive. Prevalences within vaccinated herds and unvaccinated herds varied substantially (Table 2).

Vaccine efficacy for uterine sample results was 98% for young animals and 90% for old animals. Vaginal sample vaccine efficacy was much lower (57% and 28%) for young and old animals, respectively. Vaccine efficacy for milk sample test results was 72% (Table 3). All logistic regression model fits and survival model fits were better than those of null models according to likelihood ratio tests.

For vaccinated animals, uterine samples from young animals were 0.5% as likely to be positive for *C. burnetii*

Table 1. Characteristics of goat and sheep farms sampled for *Coxiella burnetii*, the Netherlands, January–April 2010*

Farm	No. animals culled	No. live animals	Vaccination period	Bulk milk sample PCR result and date of change, 2010†
Unvaccinated goats				
A	550	178	NA	+
B	102	530	NA	Mar
F	53	938	NA	Mar
K	121	649	NA	Feb
L	324	367	NA	+
Unvaccinated sheep				
X	128	378	NA	Jan
Vaccinated goats				
H	365	673	2009 Aug–Dec	Jan
M	719	3,557	2009 Dec–2010 Jan	+
P	625	1,750	2009 Sep–Dec	+
Q	685	281	2009 Aug–Oct	+
R	3,595	0	2009 Sep–Oct	+
S	180	358	2009 Oct	+
T	1,081	83	2009 Apr–Sep	+

*Data from Animal Health Service and the Food and Consumer Product Safety Authority. Animals were vaccinated with Coxevac (Ceva Santé Animale, Libourne, France). No. live animals is the number of nonpregnant animals remaining after culling. NA, not applicable; +, positive.
†Shown are farms that had a positive PCR result at the start of the culling period (+) and those for which a PCR result changed from negative to positive during the culling period (date).

Table 2. Quantitative PCR results and prevalence for samples positive for *Coxiella burnetii* for 957 animals in 13 small ruminant herds, t January–April 2010*

Farm	Group	Uterine fluid				Vaginal mucus				Milk			
		No.	Pos	D	% (95% CI)	No.	Pos	D	% (95% CI)	No.	Pos	D	% (95% CI)
Unvaccinated goats													
A	Young	46	0	0	0 (0–6)	0	0	0	NA	0	0	0	NA
	Old	47	0	2	0 (0–6)	0	0	0	NA	52	8	2	15 (6–25)
B	Young	74	35	16	47 (36–59)	76	75	0	99 (96–100)	0	0	0	NA
	Old	26	10	2	39 (20–57)	26	26	0	100	26	17	8	65 (47–84)
F	Young	49	35	4	71 (59–84)	53	52	0	98 (95–100)	0	0	0	NA
	Old	0	0	0	NA	0	0	0	NA	0	0	0	NA
K	Young	26	17	5	65 (47–84)	32	32	0	100	0	0	0	NA
	Old	28	12	3	43 (25–61)	39	39	0	100	34	33	0	97 (91–100)
L	Young	37	0	0	0 (0–8)	37	2	9	5 (0–13)	0	0	0	NA
	Old	58	0	0	0 (0–5)	58	1	3	2 (0–5)	51	2	3	4 (0–9)
Unvaccinated sheep													
X	Young	17	5	2	29 (8–51)	17	17	0	100	0	0	0	NA
	Old	79	11	13	14 (6–22)	82	76	1	93 (87–98)	79	19	18	24 (15–34)
Vaccinated goats													
H	Young	48	1	0	2 (0–6)	49	1	5	2 (0–6)	0	0	0	NA
	Old	50	0	0	0 (0–6)	50	6	11	12 (3–21)	37	0	0	0 (0–8)
M	Young	50	0	1	0 (0–6)	49	46	2	94 (87–100)	0	0	0	NA
	Old	47	1	3	2 (0–6)	48	47	0	98 (94–100)	47	5	12	11 (2–20)
P	Young	0	0	0	NA	0	0	0	NA	0	0	0	NA
	Old	0	0	0	NA	30	12	9	40 (23–58)	30	1	0	3 (0–10)
Q	Young	49	0	0	0 (0–6)	50	2	8	4 (0–9)	0	0	0	NA
	Old	49	0	1	0 (0–6)	50	2	12	4 (0–9)	50	0	2	0 (0–6)
R	Young	0	0	0	NA	0	0	0	NA	0	0	0	NA
	Old	10	0	0	0 (0–26)	0	0	0	NA	10	0	0	0 (0–26)
S	Young	46	0	0	0 (0–6)	50	4	6	8 (0–16)	0	0	0	NA
	Old	25	0	0	0 (0–11)	28	2	5	7 (0–17)	28	1	5	4 (0–10)
T	Young	49	0	0	0 (0–6)	0	0	0	NA	0	0	0	NA
	Old	47	0	0	0 (0–6)	0	0	0	NA	46	3	3	7 (0–14)

*No., no. tested; pos, no. with positive result; D, no. with doubtful result; %, prevalence; CI, confidence interval; young, nulliparous; NA, not applicable; old, pregnant and lactating.

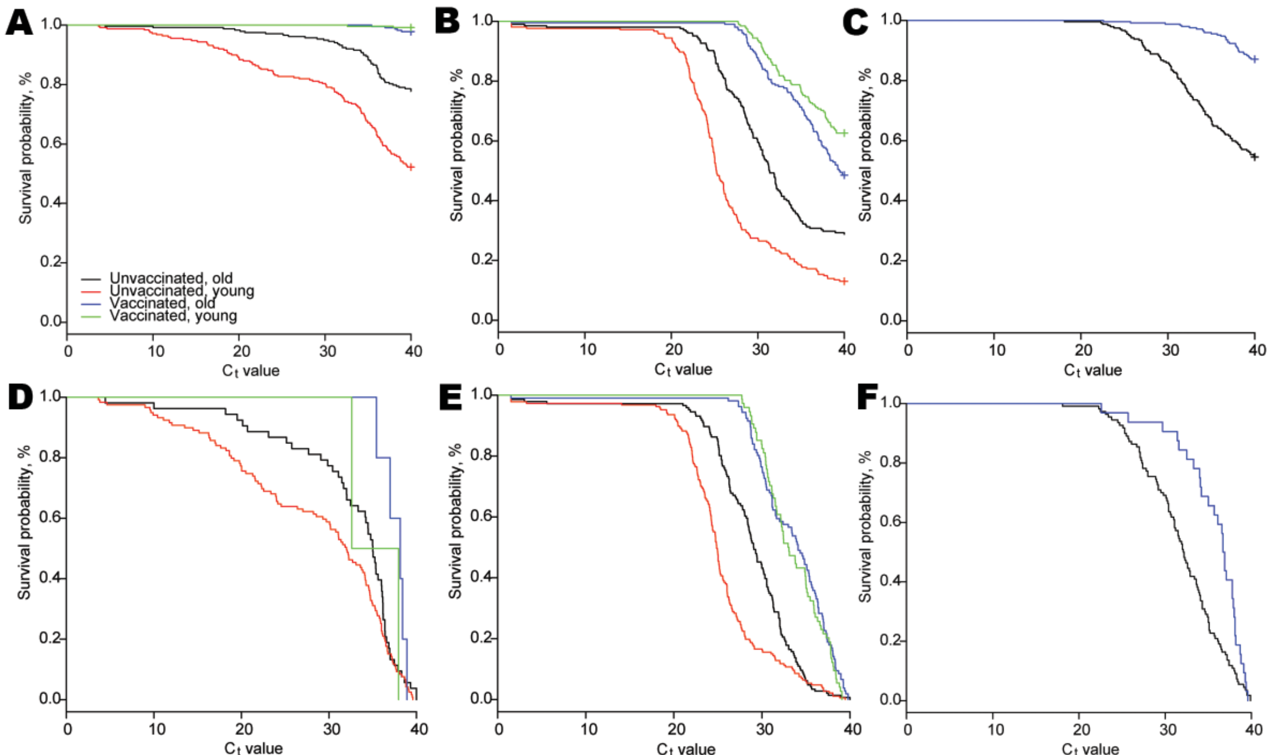

Figure 2. Kaplan-Meier curves for cycle threshold (C_t) values of all samples (A–C) and for samples with positive and doubtful results for *Coxiella burnetii* (C_t ≤40) (D–F), the Netherlands, January 1–June 10, 2009. A, D) Uterine fluid; B, E) vaginal mucus; C, F) milk. Old, pregnant and lactating; young, nulliparous.

(OR 0.005, 95% CI 0.0002–0.1200), and uterine samples from old animals were 3.2% as likely to be positive (OR 0.032, 95% CI 0.002–0.580) than samples from unvaccinated young animals. For unvaccinated animals, old animals were 44% as likely to be positive than young animals (OR 0.44, 95% CI 0.25–0.78) (Table 4). Results from the vaginal swabs were comparable; vaccinated young animals were 1.5% as likely to be positive for *C. burnetii* than unvaccinated young animals (OR 0.015, 95% CI 0.0006–0.3500). Milk from vaccinated old animals was 4% as likely to be positive for *C. burnetii* than milk from unvaccinated old animals (OR 0.04, 95% CI 0.003–0.380) (Table 5).

Effect of Vaccination on C_t Value

In uterine fluid, vaccinated animals had an HR that was half that of unvaccinated animals (HR 0.49, 95% CI 0.34–0.70), which indicated that unvaccinated *C. burnetii*–positive animals had higher relative amounts of bacteria on the basis of C_t value. This effect was similar for vaginal mucus (HR 0.34, 95% CI 0.28–0.42) and milk (HR 0.54, 95% CI 0.39–0.75) (Table 6).

Table 3. Efficacy of vaccination against *Coxiella burnetti* for 957 animals in 13 small ruminant herds, the Netherlands, January–April 2010*

	Uterine fluid				Vaginal mucus				Milk			
Group	No.	Pos	D	E, %	No.	Pos	D	E, %	No.	Pos	D	E, %
Unvaccinated												
Young	249	92	27	NA	215	178	9	NA	NA	NA	NA	NA
Old	238	33	20	NA	205	142	4	NA	242	79	31	NA
Subtotal	487	125	47	NA	420	320	13	NA	242	79	31	NA
Vaccinated												
Young	241	1	1	98	198	53	21	57	NA	NA	NA	NA
Old	228	1	4	90	206	69	37	28	248	10	22	72
Subtotal	470	2	5	NA	404	122	58	NA	248	10	22	NA
Total	957	127	52	NA	824	442	71	NA	490	89	53	NA

*No., no tested; pos, no. with positive result; D, no. doubtful; E, vaccine efficacy; young, nulliparous; NA, not applicable; old, pregnant and lactating.

Table 4. Multivariate logistic regression of prevalence of *Coxiella burnetii* in culled animals from 13 small ruminant herds, the Netherlands, January–April 2010*

	Uterine fluid		Vaginal mucus	
Group	OR (95% CI)	p value	OR (95% CI)	p value
Unvaccinated				
Young	1	NA	1	NA
Old	0.44 (0.25–0.78)	<0.05	0.22 (0.08–0.64)	<0.05
Vaccinated				
Young	0.005 (0.0002–0.12)	<0.05	0.015 (0.0006–0.35)	<0.05
Old	0.03 (0.002–0.58)	<0.05	0.13 (0.006–3.01)	0.2

*A random herd effect was included. OR, odds ratio; CI, confidence interval; young, nulliparous; NA, not applicable; old, pregnant and lactating.

C_t values for uterine fluid and vaginal mucus were lowest for *C. burnetii*–positive, unvaccinated young animals, which suggested that they had the highest relative amount of bacteria (Figure 2). C_t values were similar in bacteria-positive vaccinated animals, regardless of parity group, which indicated lower but similar shedding levels in all vaccinated animals. For milk samples, C_t values were lower for unvaccinated animals than for vaccinated animals.

Discussion

This study showed that vaccination of dairy goats against Q fever with Coxevac reduced the percentage of animals in which bacteria were detected and bacterial load in uterine fluid, vaginal swabs, and milk. Reduced prevalence was most prominent in uterine fluid and in young animals. Because shedding of bacteria may be quantitatively highest during parturition, abortion, and subsequent periods, these results suggest that vaccination may reduce environmental contamination, thereby contributing to reduction of risk for human exposure and associated human cases of Q fever.

Our findings are consistent with those of other studies. In a clinical trial of cattle, Guatteo et al. (*20*) demonstrated that vaccine was effective in reducing the probability of becoming a bacterial shedder when given to uninfected animals before pregnancy. Arricau-Bouvery et al. (*21*) showed that vaccination of 17 goats in a clinical trial decreased excretion of *C. burnetii*. Rousset et al. (*19*) conducted a field study of a goat herd infected with *C. burnetii* and found that vaccination did not prevent shedding but did reduce bacterial load in vaginal swabs of primiparous animals.

Although these studies provided useful data on the effect of vaccination, these data were based on a limited number of observations. The advantages of our study were that it was based on a larger number of field samples (957 animals from 13 herds) obtained from animals vaccinated under field conditions and that it tested uterine fluid, which is likely to be a good proxy for shedding at the time of kidding. A disadvantage of our study was its observational nature, in which vaccination was not conducted randomly at the herd, animal, parity, or infection levels, as would have been conducted in a clinical trial.

Table 5. Univariate logistic regression of prevalence of *Coxiella burnetii* in milk samples from culled animals in 13 small ruminant herds, the Netherlands, January–April 2010*

Group	OR (95% CI)	p value
Old, unvaccinated	1	NA
Old, vaccinated	0.04 (0.003–0.38)	<0.05

*A random herd effect was included. OR, odds ratio; CI, confidence interval; old, pregnant and lactating; NA, not applicable.

In unvaccinated herds *C. burnetii* was detected more often in uterine fluid of young animals than in old animals. However, no parity difference was observed for vaccinated herds. Rousset et al. (*19*) observed a reduced bacterial load in vaginal swabs in primiparous goats only. We also observed that the bacterial load was most reduced in young vaccinated animals. However, vaccinated young and old animals had similar bacterial loads in uterine fluid and vaginal mucus (Figure 2). Our results suggest that vaccination is more protective in nulliparous animals than in parous animals. Further investigations are required to determine whether the association between vaccination and bacterial shedding depends on vaccination before a first or subsequent pregnancy or on vaccination before or after natural exposure, and to elucidate underlying mechanisms.

As reported by Guatteo et al. (*20*), the time of vaccination before or during breeding may affect its effectiveness. In our study, whether all animals had been vaccinated before breeding was not known. On 1 farm, all animals were vaccinated after breeding, and most vaccinated animals with a positive test result for *C. burnetii* came from this farm. When we excluded this farm from the analyses, we observed a stronger effect of vaccination, which indicated that the effect of vaccination could have been underestimated.

However, the efficacy of vaccination may also have been overestimated. With exception of the dairy sheep farm, all unvaccinated herds with a high prevalence of *C. burnetii*–positive uterine samples had no known history of Q fever until milk PCR results became positive during the culling period. This result suggested a recent introduction of the infectious agent. In other unvaccinated herds that had only a few positive uterine samples, *C. burnetii* was

Table 6. Univariate survival analysis of PCR C_t values for *Coxiella burnetii* in positive and doubtful samples from culled animals in 13 small ruminant herds, the Netherlands, January–April 2010*

	Uterine fluid		Vaginal mucus		Milk	
Group	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value
Unvaccinated	1	NA	1	NA	1	NA
Vaccinated	0.49 (0.39–0.70)	<0.05	0.34 (0.28–0.42)	<0.05	0.54 (0.39–0.75)	<0.05

*C_t, cycle threshold; HR, hazard ratio; CI, confidence interval; NA, not applicable.

circulating before the culling period. All vaccinated herds appeared to have histories of *C. burnetii* infection. This factor makes it difficult to conclude whether absence of positive uterine samples in vaccinated herds was caused by vaccination or was a combined effect of vaccination and an immune response after natural infection.

Another study limitation is that the stage of pregnancy can affect the amount of *C. burnetii*; bacterial load in secreta may increase sharply during the last stage of pregnancy (*4*). Although we attempted to select animals that were closest to giving birth, not all animals sampled were in the same stage of pregnancy, and the average duration of pregnancy may have differed from farm to farm. Because data about gestation stage were lacking, we did not include this factor in our analyses.

Goats and sheep in the Netherlands were vaccinated to reduce the number of human cases of Q fever. However, other countries use a different strategy. In Australia, persons at risk are vaccinated against Q fever (*29*). In France, cattle are vaccinated to prevent economic losses caused by abortions (*30*). No substantial numbers of human cases of Q fever have been reported in these countries (*31*). The effect of vaccination in the Netherlands on reduction of human exposure could not be quantified. However, the low number (≈350) of human cases in 2010 compared with those in 2009 (*32*) suggests a beneficial effect of intervention measures. The relationship between bacterial shedding, environmental contamination, and human cases needs further investigation.

Our results showed that in uterine fluid, vaginal mucus, and milk, *C. burnetii* prevalence and load were reduced in vaccinated animals in the Netherlands. These effects were most pronounced in young primiparous animals. We can reasonably assume that vaccination under field conditions contributed to reduction of shedding of *C. burnetii* by dairy goats and dairy sheep, which in turn may contribute to reduction of the risk for human exposure.

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Dr Hogerwerf is a veterinarian and disease ecologist at Utrecht University. Her research interests are the epidemiology and ecology of emerging zoonoses, including Q fever.

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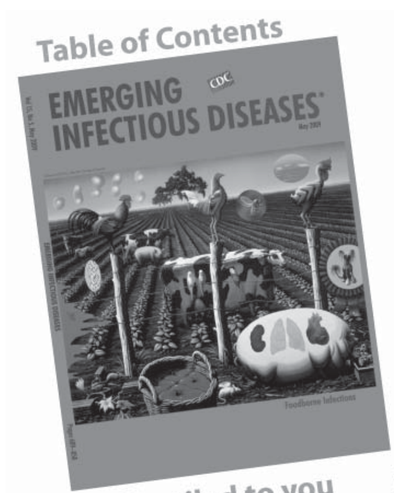

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Serologic Surveillance of Anthrax in the Serengeti Ecosystem, Tanzania, 1996–2009

Tiziana Lembo, Katie Hampson, Harriet Auty, Cari A. Beesley, Paul Bessell, Craig Packer, Jo Halliday, Robert Fyumagwa, Richard Hoare, Eblate Ernest, Christine Mentzel, Titus Mlengeya, Karen Stamey, Patricia P. Wilkins, and Sarah Cleaveland

Bacillus anthracis, the bacterium that causes anthrax, is responsible for varying death rates among animal species. Difficulties in case detection, hazardous or inaccessible carcasses, and misdiagnosis hinder surveillance. Using case reports and a new serologic assay that enables multispecies comparisons, we examined exposure to and illness caused by *B. anthracis* in different species in the Serengeti ecosystem in Tanzania during 1996–2009 and the utility of serosurveillance. High seroprevalence among carnivores suggested regular nonfatal exposure. Seropositive wildebeest and buffalo showed that infection was not invariably fatal among herbivores, whereas absence of seropositivity in zebras and frequent detection of fatal cases indicated high susceptibility. Exposure patterns in dogs reflected known patterns of endemicity and provided new information about anthrax in the ecosystem, which indicated the potential of dogs as indicator species. Serosurveillance is a valuable tool for monitoring and detecting anthrax and may shed light on mechanisms responsible for species-specific variability in exposure, susceptibility, and mortality rates.

Anthrax, which is caused by the gram-positive, sporulating bacterium *Bacillus anthracis*, primarily affects herbivorous livestock and wildlife species, but also poses serious public health risks in many parts of the world. Carnivores may also become infected by ingesting contaminated carcasses, but disease-associated illness and death are rarer than in herbivores. Although the multihost nature of the pathogen presents epidemiologic challenges, heterogeneities in host range and infection outcome provide opportunities for disease surveillance, e.g., through the use of sentinel or indicator species to detect the pathogen and changes in its prevalence or incidence (*1*,*2*).

Despite the recognized value of serologic data for disease surveillance and epidemiologic investigations, serologic analysis has only rarely been used in studies of anthrax. One reason may be the perception that case detection is relatively straightforward: a syndrome of sudden death in herbivores is useful for presumptive diagnosis, and microscopic examination of blood smears provides a relatively simple confirmatory test. However, in many environments in which anthrax is endemic, carcasses deteriorate rapidly, are hazardous, and may be inaccessible for sampling for laboratory confirmation. The utility of carcasses for case detection depends on the likelihood of observation and subsequent reporting (*3*). In many parts of Africa, anthrax is typically documented only during large, dramatic outbreaks (*4*–*7*). In remote areas or during small outbreaks, carcasses often go undetected and, even when detected, may provide only an incomplete picture of spatiotemporal patterns of infection. In humans, many anthrax cases are not reflected in hospital records. Underreporting is particularly likely for pulmonary and gastrointestinal anthrax, which have high case-fatality rates and pose diagnostic challenges (*8*,*9*), leading to a lack of appreciation of the true scale of the disease in anthrax-endemic regions.

Another explanation for the lack of serologic studies may be the perception that because sudden death is a distinctive feature of anthrax in herbivores, most infected

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animals will not survive to produce an antibody response. However, susceptibility varies widely among species, and even within a susceptible species, seropositive animals have been detected, e.g., in cattle in the United Kingdom (*10*) and bison (*Bison bison*) species in North America (*11*). Turnbull et al. documented serologic evidence of infection in Etosha lions (*Panthera leo*) and suggested that lions can serve as an indicator species of anthrax in disease-endemic areas because of their territorial behavior (*10*,*12*). The potential of using serologic analysis for comparative studies has also been limited because, up until now, serologic assays for detecting antibodies to anthrax in a variety of species have not been widely available, even in research settings.

The constraints of anthrax surveillance, particularly in tropical areas, highlight the need to identify alternative approaches to overcome these difficulties. We present results of analyses of data obtained opportunistically in the Serengeti ecosystem in Tanzania to explore the value of serologic analysis for providing information about anthrax infection and exposure patterns in large, remote, and complex ecosystems. Using seroprevalence data obtained with 1 assay for a range of species, we also investigate within-species and between-species variations in exposure and survival to evaluate which species may be useful as indicators of anthrax for surveillance purposes, specifically to identify high-risk areas for human and livestock populations.

Materials and Methods

Study Area

The study area in the Serengeti ecologic region in northwestern Tanzania comprised wildlife-protected areas, including the Serengeti National Park (SNP) and adjacent game reserves (Maswa, Ikorongo, and Grumeti). Study sites also included multiethnic, agropastoralist communities west of SNP and Ngorongoro District east of SNP, a multiple land use game controlled area inhabited by low-density Maasai and Sonjo communities that had production systems based on traditional pastoralism and limited cultivation. Ngorongoro district is divided into the Loliondo Game Control Area in the north and the Ngorongoro Conservation Area (NCA) in the south (Figure 1).

Disease Monitoring Operations

Disease detection in wildlife was based upon passive surveillance operations in the study area during 1996–2009. Sightings of carcasses were reported through a network of veterinarians from Tanzania National Parks (TANAPA) and Tanzania Wildlife Research Institute (TAWIRI), rangers, scientists, and tour operators.

Passive surveillance data from outside SNP were available through veterinary office records (government offices, TAWIRI, TANAPA, and NCA Authority). Human anthrax cases were compiled from records of government and mission hospitals in the study area for 1995–2008. Further information about human anthrax cases was obtained through key informants in villages affected by a major anthrax outbreak in 2006, and data on the age, sex,

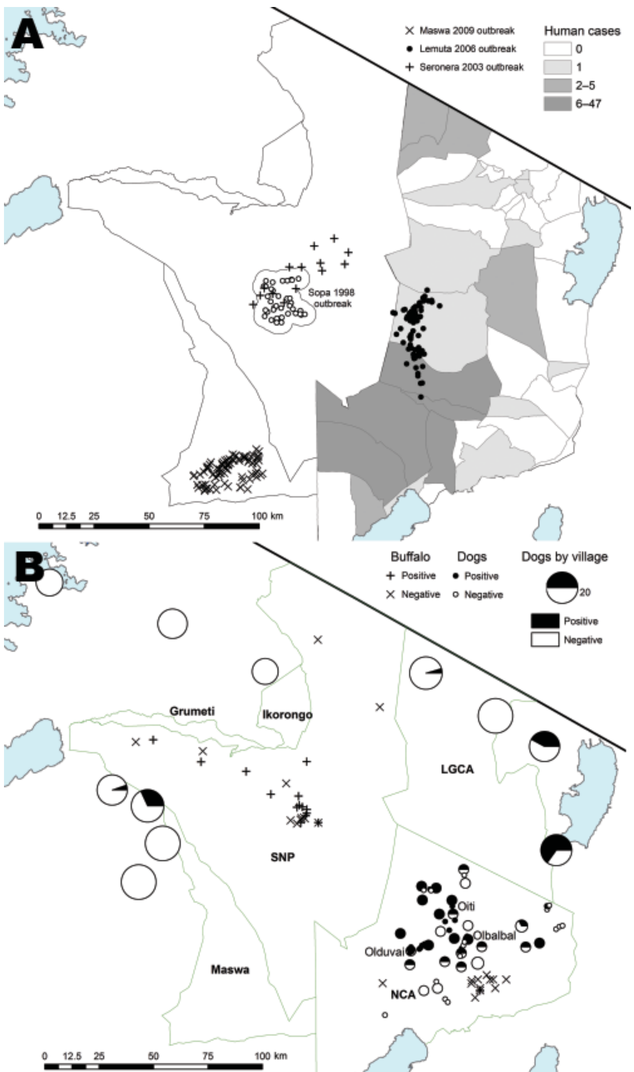

Figure 1. Anthrax cases and exposure to anthrax in the study area, Tanzania. Blue areas indicate lakes. A) Location of wildlife carcasses during anthrax outbreaks. Shaded areas indicate regions where human anthrax cases were reported during 1995–2008. Exact locations of carcasses obtained during the Sopa 1998 outbreak were not available; open circles indicate area where 549 probable cases and 67 suspected cases were detected. For the Seronera 2003 outbreak, locations of cases were randomized within a 10-km radius of the outbreak area because exact locations of carcasses were not available. B) Seroprevalence in domestic dog populations from sampled villages. Sample size is indicated by the radii of the pie charts. Green border indicates Serengeti ecosystem. LGCA, Loliondo Game Control Area; SNP, Serengeti National Park; NCA, Ngorongoro Conservation Area.

clinical presentation, and outcome of infection of affected family members was obtained from heads of affected households. No human cases were confirmed by laboratory diagnostic tests.

Case Definitions

Because of the relatively low proportion of suspected cases from which diagnostic samples were obtained, cases were classified as suspected for carcasses found that had no obvious cause of death, and probable for carcasses that showed evidence of bloody discharge from the anus, vulva, nostrils, mouth, eyes, or ears and incomplete rigor mortis. Microscopic examination of methylene blue–stained blood smears was conducted for some carcasses. Smears positive by microscopy (detection of encapsulated bacilli) were combined with probable cases (27% of probable cases were confirmed by microscopy) for analyses. No samples were confirmed by bacterial culture and isolation because of lack of facilities locally. Because of abundant scavengers in the ecosystem and logistical challenges of surveillance over such a large and remote area, many cases will go undetected on the basis of our definitions. Specifically, our definitions comprised only intact or partially scavenged carcasses; this second group also included carcasses obtained during major reported outbreaks. Carcasses that were too decomposed or scavenged to enable classification were not included in either group.

Serum Samples

Serum samples were obtained opportunistically as part of long-term epidemiologic and ecologic studies. Samples were obtained from 3 groups. The first group comprised wild carnivore species, including 263 Serengeti and 23 Ngorongoro Crater lions obtained during 1985–2007 and 53 Serengeti spotted hyenas (*Crocuta crocuta*) obtained during 1998–2007. The second group comprised Serengeti and Ngorongoro Crater ungulate species (28 and 21 buffalo [*Syncerus caffer*], 36 and 23 wildebeest [*Connochaetes taurinus*], and 74 and 11 zebra [*Equus burchellii*], respectively) obtained during 1998–2007. The third group comprised domestic dog (*Canis familiaris*) populations living adjacent to protected areas (4 villages in Loliondo Division, 13 in NCA, and 7 from areas west of SNP), which included 169 random samples obtained as part of other epidemiologic surveys and 53 samples linked to a major anthrax outbreak in pastoralist areas in 2006.

Serologic Assays

The QuickELISA Anthrax-PA Kit immunoassay (Immunetics Inc., Boston, MA, USA) was used to detect antibodies against protective antigen (PA) of *B. anthracis* in serum samples (*13*). The assay detects immunoglobulins in a subtype-independent and species-independent manner. The assay was conducted according to the manufacturer's instructions. Briefly, serum (10 μL) was incubated with a mixture of 2 recombinant PA (rPA) conjugates: streptavidin-rPA and horseradish peroxidase–rPA. PA-specific, multivalent antibodies formed ternary complexes in which streptavidin-rPA and horseradish peroxidase–rPA were bound to different antigen-combining sites on 1 antibody molecule. Complexes were bound to the biotin-coated microplates by using streptavidin conjugate and detected by using horseradish peroxidase conjugate.

Bound antibodies against PA were detected by using a chromogenic peroxidase substrate (tetramethylbenzidine). The color development reaction was stopped by addition of 2N sulfuric acid. Absorbance at 450 nm, corrected by a 620–650 nm background subtraction, was measured by using an ELISA microplate reader. Interpretation was based on comparison of absorbance for the sample with an assay-defined cutoff value. The QuickELISA Anthrax-PA kit was configured to detect ≈300 ng/mL of PA-specific antibody at the cutoff value. The assay cutoff value was defined as the mean net absorbance at 450 nm plus 0.1 of the negative control (provided in the kit); the targeted cutoff range was 0.11–0.25.

Data Analysis

A generalized linear modeling framework was used to investigate seroprevalence patterns in domestic dogs and wildlife with a binary outcome (seronegative or seropositive) and binomial error structure. Three models were constructed. First, we tested for overall species-specific differences in seroprevalence among lion, hyena, buffalo, wildebeest, and zebra populations in SNP and Ngorongoro Crater. Second, we tested for differences in seroprevalence in lion and hyena populations between years. Third, we analyzed seroprevalence patterns in domestic dogs in relation to their age and geographic location; village was modeled as a random effect by using a generalized linear mixed model.

Results

Case-Detection Patterns

Wildlife Cases

Potential anthrax cases, i.e., suspected and probable cases, were detected in a wide range of wildlife species, including wildebeest, buffalo, impala (*Aepyceros melampus*), giraffe (*Giraffa camelopardalis*), Thomson's gazelle (*Eudorcas thomsonii*), and Grant's gazelle (*Nanger granti*), hippopotamus (*Hippopotamus amphibius*), elephant (*Loxodonta africana*), and topi (*Damaliscus korrigum jimela*) (Table 1). The anthrax-attributed deaths of 1 cheetah (*Acinonyx jubatus*) and 1 serval cat (*Leptailurus*

Table 1. Potential anthrax cases detected in Serengeti wildlife species, Tanzania, 1996–2009

Common name	Species	No. cases	
		Suspected	Probable
Baboon	*Papio anubis*	0	1
Black rhinoceros	*Diceros bicornis*	1	0
Buffalo	*Syncerus caffer*	20	85
Bushbuck	*Tragelaphus scriptus*	1	0
Cheetah	*Acinonyx jubatus*	0	1
Duiker	*Sylvicapra grimmia*	0	1
Eland	*Taurotragus oryx*	0	1*
Elephant	*Loxodonta africana*	24	7
Grant's gazelle	*Nanger granti*	2	3
Giraffe	*Giraffa camelopardalis*	14	8
Hartebeest	*Alcelaphus buselaphus*	0	1
Hippopotamus	*Hippopotamus amphibious*	32	14
Impala	*Aepyceros melampus*	35	659
Ostrich	*Struthio camelus*	0	1
Serval	*Leptailurus serval*	0	1
Thomson's gazelle	*Eudorcas thomsonii*	7	5
Topi	*Damaliscus korrigum jimela*	0	4
Vulture	*Gyps africanus*	0	2
Warthog	*Phacochoerus africanus*	0	2
Waterbuck	*Kobus ellipsiprymnus defassa*	1	1
Wildebeest	*Connochaetes taurinus*	112	60
Zebra	*Equus burchellii*	34	83

*Outbreak reported but individual case counts not available.

serval) during an outbreak in 1998 were the only carnivore cases reported during the study.

Cases were detected every year in NCA and SNP. However, major reported outbreaks were limited in time and space (Figure 1, panel A). Species affected and extent of outbreaks varied. Impalas were predominantly affected during 2 outbreaks (in southcentral Serengeti in late January–early February 1998 [*14*] and in central Serengeti in January 2003). Wildebeest and zebras were mostly affected during an outbreak east of SNP in early 2006. Buffalo were most recently affected southwest of SNP in October 2009. Small numbers of zebra deaths were recorded regularly throughout the study period, in addition to the 2006 outbreak.

Livestock Cases

During 1996–1999, several large outbreaks (>500 deaths) were documented in livestock (goats, sheep, and cattle) east of SNP. Suspected cases were reported regularly in some localities in apparently localized disease-endemic foci (i.e., Olbalbal, Oiti, and Olduvai; Figure 1, panel B). Small-scale vaccinations in the local vicinity were generally performed in response to these outbreaks. No livestock cases were reported from 2000 until the end of 2003, during which time considerable livestock vaccination was conducted. Livestock cases have been reported since 2004, and many livestock carcasses were obtained during the 2006 wildlife outbreak (Figure 2). However, local Maasai communities attributed these deaths to starvation, and diagnostic material was not available for confirmation.

Human Cases

Hospital records of anthrax-infected humans were typified by sporadic reports of nonfatal cutaneous anthrax from a few localities (Figure 1, panel A; Figure 2). However, small-scale household questionnaire surveys conducted in villages where livestock and wildlife anthrax outbreaks had occurred indicated several cases in persons who had eaten affected livestock carcasses (50% case-fatality rate, 4 deaths and 8 cases in 7,538 persons). Clinical signs included diarrhea and swollen abdomen (consistent with ascites), which are features of gastrointestinal anthrax.

Serologic Patterns

Wildlife

Seroprevalence patterns of sampled wildlife varied widely and showed differences among species and populations (Figure 3). Overall seroprevalence was lower in wildlife populations in Ngorongoro than in Serengeti ($p<0.001$). Seroprevalence was consistently high in wild carnivores (90% and 57% overall seropositivity in Serengeti and Ngorongoro Crater lions, respectively, and 87% seropositivity in Serengeti spotted hyenas) and did not show any significant year-to-year variation, e.g., in years of known outbreaks ($p>0.05$). Age seroprevalence in Serengeti lions indicated a high frequency of infection,

seroconversion at a young age, and seropositive animals in all age groups (Figure 4, panel A). Seroprevalence was lower among herbivorous species (46% and 14% seropositivity in Serengeti and Ngorongoro Crater buffalo and 19% and 4% in Serengeti and Ngorongoro Crater wildebeest); no zebras were found to be seropositive despite relatively extensive sampling efforts (Figure 3).

Domestic Dogs

Seroprevalence patterns in domestic dog populations showed marked regional differences (Figure 1, panel B). Low seroprevalence was observed in agropastoralist western communities, and high and spatially variable seroprevalence was observed in pastoralist eastern communities ($p<0.001$) (Figure 1, panel B; Table 2). More specifically, high seroprevalence was recorded in dogs in pastoralist areas where livestock cases had been regularly reported (e.g., Olbalbal, Oiti, and Olduvai areas). High seroprevalence was also observed in domestic dogs sampled 6 weeks after the outbreak was detected in zebras and wildebeest in Ngorongoro in early 2006 (Figure 4, panel B), and changes in mean antibody levels in domestic dogs sampled before and after the outbreak also reflected recent exposure (Figure 2). Most dogs sampled in western areas of the Serengeti were seronegative, which is consistent with an absence of reports of anthrax cases in either the veterinary office or hospital records. However, seropositive dogs were detected in 1 village, Gibeshi, where anthrax had not been previously recorded (Figure 1, panel B). Age seroprevalence patterns of dogs sampled in 1999 indicated seropositivity only in dogs >1 year of age, which is consistent with exposure occurring in 1997 or 1998 (Figure 4, panel B).

Discussion

We report wide variation in patterns of exposure to anthrax and deaths among wild and domestic animal species and populations of the Serengeti ecosystem. Serologic data also highlight the potential value of domestic dogs as indicator species for identifying high-risk areas of infection for livestock and human populations.

The QuickELISA Anthrax-PA kit was a convenient method for assessing seroprevalence in the multiple species examined in this study. The assay does not rely on species-specific or protein A/G conjugates to detect anthrax-specific antibodies and thus can detect any multivalent antibody in a sample. Previous studies that examined antibodies against anthrax in wildlife required unique conjugates specific for each species studied, which necessarily limited the number of species that could be examined (*10*). In our study, antibodies were measured in 6 species by using 1 assay. Thus, relative amounts of antibody present in each sample could be directly compared.

Lack of obvious clinical signs before death, inaccessibility of remote locations, decomposition, and hazardous carcasses all affect the quality of anthrax surveillance based on case detection. Despite concerted efforts to obtain samples from suspected cases, we recovered little diagnostic material for confirmation. However, more probable cases were identified on the basis

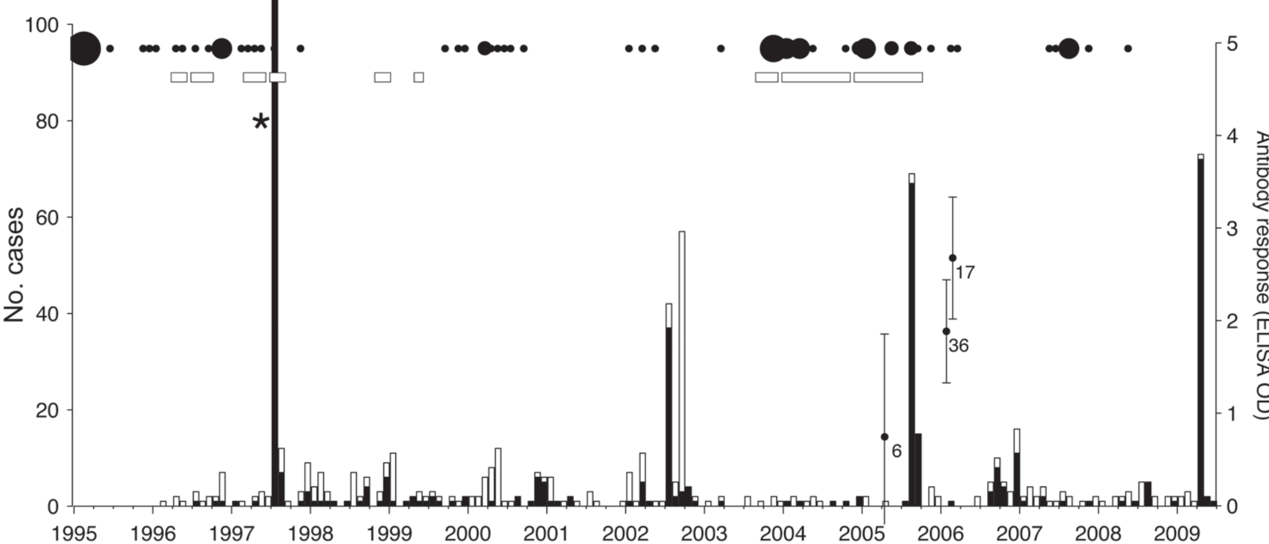

Figure 2. Anthrax case detection in wildlife, livestock, and human populations in the Serengeti ecosystem, Tanzania, 1996–2009. Probable (black bars) and suspected (white bars) wildlife cases (as defined in the Materials and Methods) are shown. Black circles indicate hospital records of anthrax scaled according to the number of cases, and rectangles indicate when cases in livestock were reported (quality of the data for livestock cases was too poor to quantify). Domestic dogs were sampled in villages near wildlife cases detected in 2006. Error bars indicate mean antibody responses and 95% confidence intervals at the time of sampling; sample sizes are indicated. *During the 1998 outbreak, 549 probable cases and 67 additional suspected cases were detected. OD, optical density.

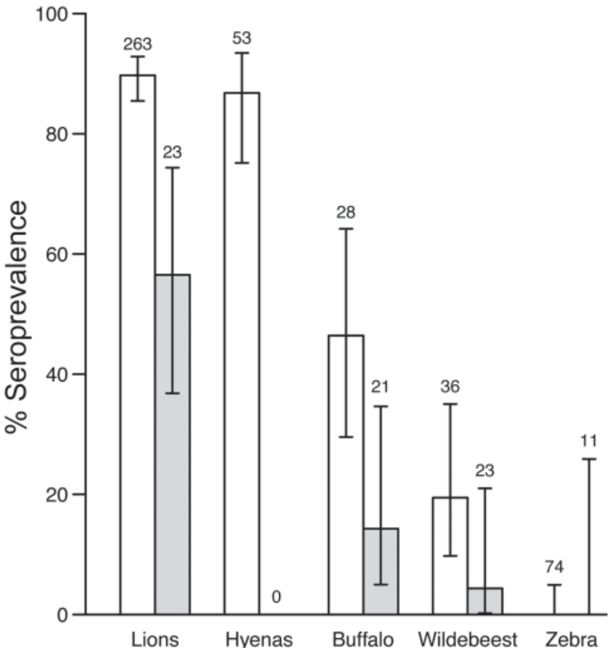

Figure 3. Seroprevalence of anthrax in sampled wildlife populations from Serengeti National Park (white bars) and Ngorongoro Crater (gray bars), Tanzania, 1996–2009. Sample sizes used to calculate seroprevalence are indicated above the bars. Hyenas were not sampled in Ngorongoro Crater. Seropositive zebras were not detected. Error bars indicate 95% confidence intervals based on a binomial distribution of the sample size and the seropositivity range that can be expected.

of the appearance of carcasses. Laboratory results may not be conclusive even when diagnostic material is obtained. For example, when multiple blood slides were prepared from 1 buffalo carcass and tested blindly, the results were not consistently positive (only 2 of 6 slides were positive). Some outbreaks are likely to be missed even when probable case detection is used. This suggestion was confirmed by using serosurveillance data; in some agropastoralist areas where anthrax had not been reported, serologic analysis of domestic dogs indicated that the disease had been present.

In contrast to sampling of suspected carcasses, serosurveillance of living animals poses no risk for anthrax infection and therefore offers an opportunity for gaining a better understanding of anthrax epidemiology, particularly in relation to patterns of infection and risk factors for exposure, susceptibility, and death. Our serologic data highlight differences between species in exposure and death, which may be explained by behavior and ecology. However, we caution that although our study suggests great potential for the use of this assay for multiple species comparisons, validation of serologic responses across a range of vaccinated species (possibly using zoo collections) would provide more definitive verification of this proposition and should be prioritized.

Low mortality rates, combined with high seroprevalence rates (always >50% and approaching 90% in Serengeti populations), suggests that wild carnivores are regularly exposed to anthrax without apparent deaths. Although high mortality rates were reported for Kruger lions after periods of low anthrax incidence (*4*,*10*), high seroprevalence rates and low mortality rates are more commonly observed, which suggests a protective immune response presumably associated with more frequent exposure (*4*,*10*). Lions and hyenas may be exposed through consumption of infected prey, but domestic dogs may also be exposed when they scavenge infected carcasses (wildlife and livestock). Low

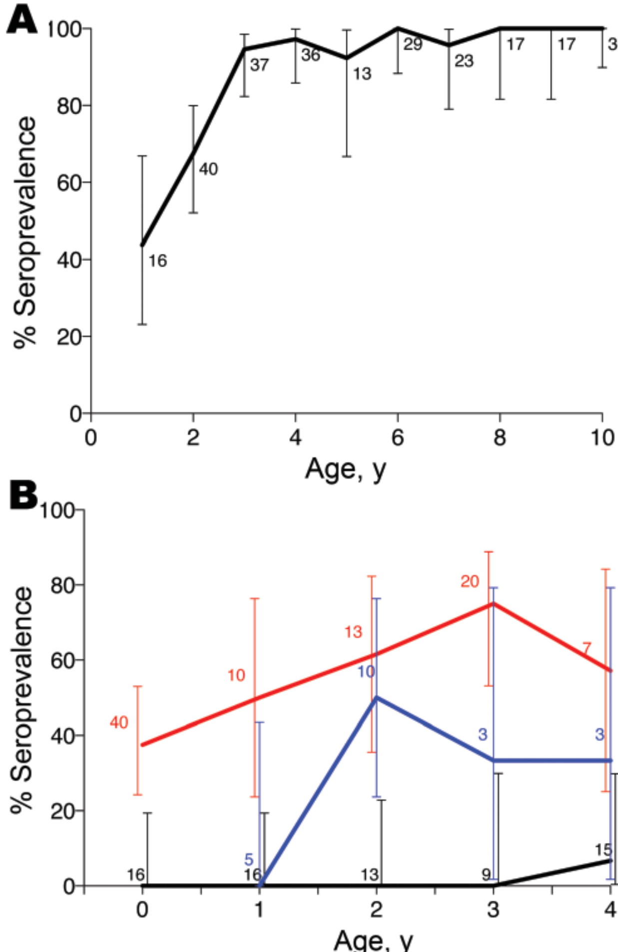

Figure 4. Anthrax seroprevalence patterns in carnivores, by age, Tanzania, 1996–2009. Lions (A) in Serengeti and domestic dogs (B) in agropastoralist regions where no outbreaks were detected (black line), in pastoralist regions where repeated outbreaks were detected (red line), and in an agropastoralist village where no outbreaks were reported but serologic surveys indicated previous exposure (blue line). Error bars indicate 95% confidence intervals for age seroprevalence in lions and dogs, but are juxtaposed for dogs to improve readability. Sample sizes used to calculate seroprevalences are indicated.

Table 2. Multivariate generalized linear mixed model analysis of risk factors for anthrax seropositivity in dogs, Tanzania, 1996–2009*

Predictor	Unit	Estimate	SE	z score	p value	OR (95% CI)
Intercept		–6.224	1.275	–4.881	<0.001	NA
Age	Months	0.043	0.011	3.956	<0.001	1.044 (1.022–1.066)
Area	West	Referent	Referent	Referent	Referent	1
	Loliondo	4.178	1.696	2.463	0.014	65.21 (2.347–1,811.000)
	NCA	4.511	1.364	3.308	<0.001	90.97 (6.283–1,317.000)

*OR, odds ratio; CI, confidence interval; NA, not applicable; NCA, Ngorongoro Conservation Area.

seroprevalence rates and high mortality rates have been reported for cheetahs (*15*,*16*). These rates are consistent with the fact that cheetahs do not scavenge, and solitary hunting exposes them to fewer carcasses than group-hunting lions and hyenas. Although data from this study are limited, detection of probable anthrax cases in cheetah and serval is consistent with a higher susceptibility in these carnivore species.

Relatively lower seroprevalence rates and higher mortality rates for ungulates than for carnivores suggest less routine exposure, higher susceptibility, or both. Major differences in herbivore susceptibility are inferred from the wide variation in seroprevalence detected in species sharing the same grazing areas, and thus probable exposure patterns. Zebras appear to be highly susceptible; however, buffalo and wildebeest can clearly survive infection. Seroprevalence in buffalo was high (≈50%) compared with previous reports for herbivores (≈7%) (*10*,*17*). Comparing seroprevalence in more species would enable assessment of relative roles of exposure versus susceptibility in explaining variable species mortality patterns characteristic for anthrax.

The reported species differences have potential implications for serosurveillance. Among wildlife, carnivores are likely to be the most sensitive indicators of whether infection is present in an area, acting as bioaccumulators of infection through consumption of infected carcasses (*18*). However, because lions and hyenas seroconvert at a relatively young age, temporal patterns of exposure from age seroprevalence data are difficult to detect. Furthermore, hyenas are highly mobile in the Serengeti ecosystem (*19*), which reduces their utility for identifying specific high-risk areas. However, more detailed investigations of titer levels in relation to timing and location of anthrax outbreaks, including longitudinal studies of serial titers from known animals, could shed light on immunologic responses and enable more information to be obtained from serologic data.

These data suggest a possible utility of serosurveillance in buffalo, whose potential as indicator species has not been explored. Because ≈50% of Serengeti buffalo are seropositive for anthrax, these populations appear to provide a relatively sensitive indicator of the presence and prevalence of anthrax infection, e.g., major differences between populations in SNP and Ngorongoro Crater. Although serologic analysis of wildebeest detected these differences, seroprevalence in wildebeest was lower overall, and the wide-ranging migratory movements of the Serengeti herds limit the utility of these data for detecting spatial patterns. In comparison, buffalo herds range over relatively restricted areas, and serologic data can pinpoint high-risk areas. In many protected areas of Africa, buffalo are already routinely sampled for surveillance of diseases, such as rinderpest, bovine tuberculosis, and foot-and-mouth disease. We suggest that in areas where buffalo surveillance is ongoing, there is added value in using serum samples for monitoring anthrax exposure patterns. Serologic analysis of buffalo and analysis of environmental risk factors could also assist wildlife management strategies, e.g., risks associated with reintroductions of rhinoceros in different areas of the Serengeti, and identify priority areas for enhanced risk-based surveillance.

Domestic dogs have high potential value as indicators of human and livestock diseases (*1*,*18*). They are regularly exposed to a wide range of infections in disease-endemic areas; they are abundant and widely distributed, especially in developing countries; they are generally accessible for safe handling and sampling; they can be sampled at young ages, which enables reasonably accurate timing of outbreaks; and they live in close association with humans and livestock, which makes them good indicators of risk. In addition, vaccination campaigns present a cost-effective opportunity for obtaining large numbers of domestic dog samples (*18*). Consistent with these expectations, we have demonstrated that dogs in the Serengeti can be useful indicators of anthrax. They can be used to detect infection in an area, even when anthrax is not identified in other species; they reflect differences in infection prevalence in different areas; they can provide information about the timing of outbreaks (we observed variation in exposure with age) (Table 2); and they serve as an indicator of livestock and human disease risk and provide a basis for risk-based surveillance and targeted implementation of prevention measures (e.g., livestock vaccination or public health campaigns).

In conclusion, we demonstrated that serologic investigations of wildlife and domestic animals can provide valuable information about patterns of anthrax transmission and for identifying areas for risk-based surveillance. Serologic approaches also enable retrospective identification of infected areas and timing of outbreaks

where case surveillance is limited because of remoteness of an area, poor reporting of cases to local or central authorities, misdiagnosis, and difficulties in performing confirmatory laboratory diagnostic tests. Further research may enable more effective use of serologic data if insights can be gained into how antibody levels relate to timing and degree of exposure.

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Mycobacterium lentiflavum in Drinking Water Supplies, Australia

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Mycobacterium lentiflavum, a slow-growing nontuberculous mycobacterium, is a rare cause of human disease. It has been isolated from environmental samples worldwide. To assess the clinical significance of *M. lentiflavum* isolates reported to the Queensland Tuberculosis Control Centre, Australia, during 2001–2008, we explored the genotypic similarity and geographic relationship between isolates from humans and potable water in the Brisbane metropolitan area. A total of 47 isolates from 36 patients were reported; 4 patients had clinically significant disease. *M. lentiflavum* was cultured from 13 of 206 drinking water sites. These sites overlapped geographically with home addresses of the patients who had clinically significant disease. Automated repetitive sequence–based PCR genotyping showed a dominant environmental clone closely related to clinical strains. This finding suggests potable water as a possible source of *M. lentiflavum* infection in humans.

Mycobacterium lentiflavum organisms are nontuberculous mycobacteria (NTM) first identified in 1996 (*1*). *M. lentiflavum* is slow growing at 22°C–37°C and has yellow pigmentation, negative tests for Tween 80 hydrolysis, nicotinic acid, nitrate reductase and urease, distinct fatty and mycolic acid patterns, and unique 16S rRNA and 65-kDa heat-shock protein gene sequences. It shares phenotypic features with *M. avium* but is more closely related to *M. simiae* and *M. genavense*. Because of similarities to *M. avium* complex (MAC), differentiation can be difficult without molecular identification, hence, misclassification in the past is possible (*2*).

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As with other NTM, *M. lentiflavum* has been isolated from soil and water samples around the world. However, links between environmental sources and human disease have not yet been demonstrated.

In Queensland, Australia (population 4.28 million), NTM disease is notifiable. A central reference laboratory performs speciation of all positive isolates. In 2008, ≈900 isolates of NTM were reported.

Strain variation within mycobacterial species is well known. Although epidemiologic studies provide useful information, molecular strain typing can be invaluable, especially if a single clone can be linked to an outbreak source. Pulsed-field gel electrophoresis (PFGE) has been considered the standard for mycobacterial strain typing but is time- and labor- intensive and requires expensive dedicated equipment. Also, DNA degradation can occur during electrophoresis, generating uninterpretable banding patterns (*3*). Repetitive sequence–based PCR (rep-PCR) has been used to differentiate mycobacterial strains associated with disease outbreaks in mesotherapy clinics (*M. abscessus* and *M. chelonae*) (*4*) and in patients after surgery (*M. fortuitum*) (*5*). An automated rep-PCR system (DiversiLab; bioMérieux, Melbourne, Victoria, Australia) showed high concordance with PFGE results (*6*) in identifying mycobacterial strain clusters and was faster than PFGE.

We had 2 goals for this study. First, we aimed to describe the clinical significance and outcomes of *M. lentiflavum* infection in Queensland. Second, we intended to explore the genotypic and geographic relationship between patient isolates and potable water isolates in the Brisbane area.

Methods

We reviewed the records of all patients from whom *M. lentiflavum* had been isolated during July 2001–November 2008. Attending physicians were contacted to establish clinical significance according to American Thoracic Society (ATS)/Infectious Diseases Society of America (IDSA) criteria (*2*) (Table 1). During 2007–2008, potable water was collected from 206 sites in Brisbane's drinking water system.

Laboratory Identification

Human samples were digested and decontaminated by using 4% NaOH, neutralized with phosphoric acid, and centrifuged to concentrate the acid-fast bacilli (AFB). Smears were prepared from the sediment and stained by the Ziehl-Neelsen (ZN) method. We injected cells into 1 Lowenstein-Jensen slope (± pyruvate) and 7-mL mycobacterial growth indicator tube, then incubated them at 35°C until growth was detected. ZN staining of colonies confirmed AFB. Multiplex PCR (*7*) was performed to discriminate between *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. abscessus*, and other *Mycobacterium* spp. Other *Mycobacterium* spp. were further speciated by using Hain Life Sciences GenoType Mycobacterium AS (additional species) kit (2004–2007 only; Hain Lifescience, Nehren, Germany) and/or 16S rRNA sequencing in conjunction with phenotypic characteristics.

Table 1. American Thoracic Society/Infectious Diseases Society of America diagnostic criteria for NTM lung disease*

Clinical criteria
Pulmonary symptoms AND
Nodular or cavitary opacities on chest radiograph OR
Multifocal bronchiectasis with multiple small nodules on high-resolution computerized tomography AND
Appropriate exclusion of other diagnoses
Microbiologic
Positive culture results from at least 2 separate expectorated sputum samples OR
Positive culture results from at least 1 bronchial wash or lavage OR
Biopsy† showing granulomatous inflammation or acid-fast bacilli *and* positive culture OR
Biopsy† showing granulomatous inflammation or acid-fast bacilli *and* one or more culture-positive sputum or bronchial washings
Comments
• Risk-benefit of therapy should be considered for each patient before institution of therapy
• Expert consultation should be obtained when NTM are recovered that are either infrequently encountered or that usually represent environmental contamination
• Patients suspected of having NTM lung disease but who do not meet the diagnostic criteria should be followed until the diagnosis is firmly established or excluded

*Adapted from (*2*). NTM, nontuberculous mycobacteria.
†Transbronchial or other lung biopsy.

Water Sampling

Water was collected from routine sampling sites across Brisbane and processed according to described methods (*8*). Each 1,000-mL sample was transported at 4°C and processed within 24 hours. Half of each sample was decontaminated by using 0.005% cetylpiridinium chloride, and each 500-mL aliquot was filtered separately by using 45-µm cellulose nitrate filters (Sartorius AG, Gottingen, Germany). The filters were rinsed and macerated in 3 mL sterile distilled water. Aliquots (0.1 mL) were transferred in triplicate to M7H11 plates, sealed in gas-permeable plastic bags, and incubated at 32°C. Aliquots (0.5 mL) were transferred to 2 mycobacterial growth indicator tubes, 1 of which contained polymyxin, azlocillin, nalidixic acid, trimethoprim, and amphotericin B. ZN staining of colonies confirmed AFB, and these colonies were subcultured on M7H11 plates. Multiplex PCR was performed (*7*) followed by 16S rRNA sequencing of mycobacterial isolates and compared by using Ribosomal Differentiation of Medical Microorganisms and GenBank databases (*9*,*10*).

Automated Rep-PCR Strain Typing

The similarity of 16 clinical and 7 water isolates was determined by using a rep-PCR method (DiversiLab). DNA was extracted from clinical and water isolates by using the Ultraclean Microbial DNA Isolation Kit (Mobio Laboratories, Carlsbad, CA, USA). PCR mixture was prepared by using AmpliTaq polymerase and PCR buffer (Applied Biosystems, Foster City, CA, USA) and Mycobacterium DiversiLab primer mix according to the manufacturer's instructions. Rep-PCR products were separated and detected by using microfluidic chips of the DiversiLab system. Fingerprints were analyzed with DiversiLab software version 3.4.38 by using the Pearson correlation coefficient and unweighted pair-group method with arithmetic means to compare isolates and determine clonal relationships.

Results

Clinical Isolates

Forty-seven isolates of *M. lentiflavum* were reported from 36 patients (Figure 1; Table 2). Full clinical information was available for 32 (89%) patients. Four patients (8 isolates) had clinically significant disease. Seven patients were taking treatment or were under surveillance for MAC (1 or 2 isolates each); no treatment changed in response to the new isolate, and thus these isolates were not considered clinically significant. Twenty-one other patients (18 adults, 3 children) had clinically nonsignificant isolates. Four patients had probable nonsignificant isolates, but sufficient clinical information was lacking. No cases demonstrated positive AFB smears by ZN staining. Of the 32 patients

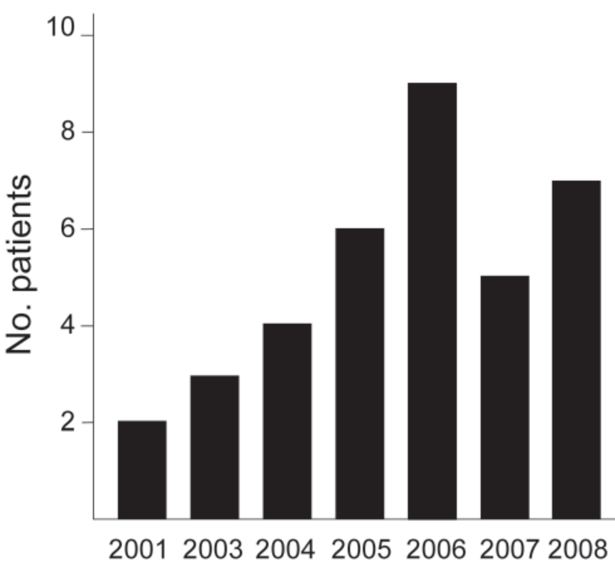

Figure 1. Number of patients from whom *Mycobacterium lentiflavum* was isolated, by year of isolation, Brisbane, Queensland, Australia, 2001–2008.

with uncertain or nonsignificant disease, 26 had 1 positive specimen, 2 had 2 positive specimens from the same period, 3 had 2 positive specimens separated by 3 months, and 1 had 2 positive specimens separated by 11 months. Antimicrobial drug susceptibility tests were performed for 2 isolates (cases 1 and 2 below). Both were sensitive to clarithromycin 4.0 µg/mL and resistant to isoniazid 0.4 µg/mL, ethambutol 5.0 µg/mL, and streptomycin 1.0 µg/mL. The case 1 isolate was sensitive to ofloxacin 2.0 µg/mL; the case 2 isolate was resistant to rifampin 1.0 µg/mL.

Environmental Isolates

Mycobacteria were grown from 70% of water sites. The predominant isolates were *M. gordonae* and *M. kansasii*. *M. lentiflavum* was isolated from 13 (6.3%) sites, 2 of which were reservoirs, 1 a treatment plant, and the remainder points in the distribution system. Eleven sites shared the same groundwater source but were distributed among 10 different reservoir zones. For 12 patients living within 20 km of Brisbane central business district, the mean distance between their residential addresses and nearest positive water site was 3.49 km (range 0.9–9.8 km). The 4 persons with clinically significant illness lived a mean of 2.7 km from a positive water site (Figure 2).

Case Descriptions for Significant Isolates

The 4 patients whose disease met the ATS/IDSA criteria are described below. All specimens were ZN stain negative.

Case 1: Disseminated Infection

A 43-year-old woman who smoked had a background of intravenous drug use and HIV. In 1998, granulomatous hepatomegaly developed, thought to be a reaction from injecting methadone mixed with orange juice, and resolved after she ceased this activity. A tunneled intravenous access device was placed in February 2006. In April 2007, she sought care for hepatosplenomegaly and mild pancytopenia. Liver and gastric lymph node biopsies showed granulomata. Two bone marrow biopsy samples taken 6 weeks apart showed initially scant, but then more marked, granulomata. All specimens were culture negative for AFB. A working diagnosis of sarcoidosis was made, and prednisone with highly active antiretroviral therapy (tenofovir, emtricitabine, and efavirenz) began. Azathioprine was introduced and prednisone ceased by April 2008. In June, she was admitted with massive hepatosplenomegaly, weight loss, and fever. CD4+ count was 0.14×10^9/L ($0.43–1.62 \times 10^9$/L), and viral load was undetectable (<50 copies/mL HIV-1 RNA). Over the next month, all 4 blood cultures grew *M. lentiflavum*; after 15 days, mycobacteria were apparent and *M. lentiflavum* was confirmed 7 days later (day 22). Bone marrow biopsy showed granulomata and grew *M. lentiflavum*. Urine and fecal samples were negative for any mycobacteria. She did not produce any sputum. Chest radiograph showed extensive miliary nodules, and computed tomography

Table 2. Characteristics of patients from whom *Mycobacterium lentiflavum* was isolated and source of isolate, Queensland, Australia, 2001–2008*

Characteristic	No. patients	Median age, y (range); sex, M/F	Source, no. isolates: Bronchial washing	Sputum	Wound swab/aspirate	Other
Adults						
Significant clinical illness	3	49 (42–85); 0/3	2	0	0	Blood, 1
Nonsignificant clinical illness	18	67 (22–88); 12/6	9	4	4	Blood, 1
Probable nonsignificant clinical illness	4	74 (59–81); 3/1	1	2	0	Ascites, 1
Nonsignificant clinical illness with MAC	7	66 (49–75); 4/3	0	7	0	0
Children						
Significant clinical illness	1	1.6; 0/1	0	0	1	0
Nonsignificant clinical illness	3	12 (1.6–17); 1/2	0	2	1	0

*MAC, *Mycobacterium avium* complex.

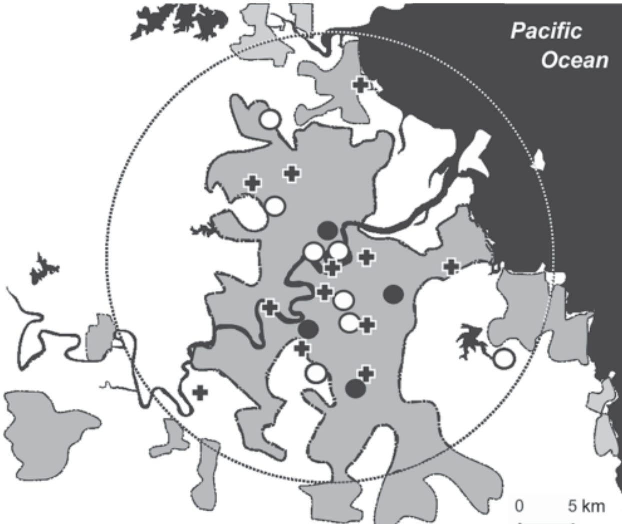

Figure 2. Urban catchment area and locations of persons and potable water from which *Mycobacterium lentiflavum* was isolated, Brisbane, Queensland, Australia, 2001–2008. Gray shading, approximate urban extent; circle, 20-km radius from central business district.

(CT) showed peribronchial thickening and bronchiolitis but no lymphadenopathy. The patient was empirically given isoniazid, rifampicin, pyrazinamide, clarithromycin, and ethambutol. Oral prednisone (25 mg 1×/d) improved symptoms and liver biochemistry and decreased splenic size. She was discharged on prednisone (15 mg 1×/d), isoniazid (300 mg 1×/d), ethambutol (400 mg 2×/d), and clarithromycin (500 mg 2×/d). Her organomegaly improved over the next 6 months. The intravenous port was removed. Ten months later, she remained well and compliant with treatment.

Case 2: Chronic Pulmonary Nodules and Bronchiectasis

In December 2007, an 85-year-old woman sought care for lobar pneumonia. She had never smoked and had no previous lung disease or immunosuppression. At follow-up after discharge from the hospital, she was lethargic with a persistent cough but no weight loss or fever. CT of her thorax confirmed bilateral well-defined nodules up to 1 cm in diameter. Bronchoscopic washings grew mycobacteria, but the organism could not be speciated. Results of a percutaneous nodule biopsy were nondiagnostic. Surgical biopsy of the right lung found caseating granulomata, but culture was negative. At 7 months follow-up, a CT scan of her thorax showed no change in the nodules, but mild bronchiectasis had developed. Bronchoscopic lavage grew *M. lentiflavum* for the first time. In February 2009, she began ethambutol (800 mg), rifampin (450 mg 1×/d), and clarithromycin (500 mg 2×/d). Her symptoms improved, and she completed 18 months of treatment. Bronchoscopic washings posttreatment were ZN stain and AFB culture negative.

Case 3: Bronchiectasis

A 49-year-old Taiwanese woman who had never smoked sought care in 1998 for hemoptysis. She had moved to Australia 5 years earlier. Thoracic CT showed a right middle lobe infiltrate. Three sputum samples were culture negative for AFB. Transbronchial lung biopsy samples showed peribronchial granulomata but were culture negative. She received empirical quadruple therapy for tuberculosis. The cough continued but without hemoptysis. In 2004, a chest radiograph showed middle lobe and lingular bronchiectasis. Three sputum samples were AFB culture negative. Bronchoscopic washings were ZN negative but grew *M. lentiflavum*, thought to represent colonization. In 2007, an unspeciated NTM grew on 1 of 3 sputum specimens. By January 2009, the patient was well, with no exacerbations in the previous year and stable radiographic appearance.

Case 4: Cervical Lymphadenitis

A 20-month-old girl was examined for a 4-week history of bilateral cervical lymphadenopathy. The largest node (20 × 24 mm) was excised. Necrotizing granulomata were seen. *M. lentiflavum* was cultured. No antimycobacterial therapy was administered; she recovered fully.

Nonsignificant Isolates

A 29-year-old woman underwent bilateral lung transplantation. Routine posttransplant bronchial washings grew *M. lentiflavum*. Despite immunosuppressive therapy, no further AFB were cultured from multiple samples in the subsequent 2.5 years.

In 7 patients (mean age 62 years, 4 male), 1 or 2 isolates of *M. lentiflavum* grew from sputum in the context of MAC disease or colonization. Four of these patients were concurrently treated for MAC; 1 had recently completed treatment; 2 received no treatment for NTM and continue under surveillance. All 7 had underlying lung disease (2 cavitatory, 5 bronchiectatic). In no instance was *M. lentiflavum* specifically treated. In addition, sputum of 3 patients grew *M. interjectum*, *M. fortuitum*, or *M .abscessus*.

From 3 otherwise healthy patients (40-year-old man, psoas abscess; 2-year-old girl, cervical lymphadenitis; 54-year-old man, neck abscess), *M. lentiflavum* and *Staphylococcus aureus* were cultured. All patients recovered fully after treatment with flucloxacillin with or without drainage. No samples were taken for histologic

examination, but cytologic examination of a lymph node aspirate from the child showed lymphocytes, macrophages, neutrophils, and fragments of epithelioid histiocytes but no well-formed granulomas. From 2 other patients (35-year-old woman, chronic leg ulcer; 59-year-old woman, post thyroidectomy wound abscess), *M. lentiflavum* without *S. aureus* were cultured; the patients were treated with wound debridement and flucloxacillin. Biopsy samples showed no granulomata.

Most other isolates were cultured from respiratory samples. One isolate each was grown from ascitic fluid and blood. Three patients with cystic fibrosis (2 with mild disease, 1 lung transplant recipient) had 1 or 2 isolates each but no evidence of disease.

Strain Types

DiversiLab patterns were grouped into 7 rep-PCR profiles, A–G (Figure 3). The 8 clinical isolates of profile A showed 97%–99% similarity. This profile included 2 clinically significant isolates (cases 1 and 3) and 6 nonsignificant isolates (3 respiratory samples, 2 soft tissue samples, and 1 ascites sample). Two further pulmonary isolates (profiles A1 and A2) were ≈90% similar to the profile A isolates. The isolate from case-patient 2 was contaminated and could not be analyzed. The isolate from case-patient 4 (profile B) had 94% similarity to a nonsignificant isolate from soft tissue. These 2 isolates were from patients who lived 1,800 km apart.

Profile D comprised a pair of nonsignificant pulmonary isolates of 97% similarity. These isolates came from patients who lived within 80 km of each other, 450 km north of Brisbane. Profiles C and E were nonsignificant isolates and distinct from other rep-PCR profiles.

Five water sample isolates (profile A3) had 97%–99% similarity and shared 90% similarity with the clinical isolates of profiles A, A1, and A2. The other 2 water isolates (profiles F and G) were distinct from all other clinical and water isolates.

Global Case Reports

In 30 cases of clinically significant disease published in English (online Appendix Table, www.cdc.gov/EID/content/17/3/395-appT.htm), disease spectrum varied from cervical lymphadenitis (8 of 9 cases in children) to acute or chronic disease usually affecting lungs and pleura (infiltrates, cavities, nodules, effusions) but also arthritis/discitis, bone lesions, skin ulcers, and hepatosplenomegaly. The rapid onset of cervical lymphadenitis has been noted in many reports, usually with an excellent outcome from excision alone. The mean age of adults with nonlymphadenitis disease (20 cases) was 56 years (range 23–87 years), and they were evenly split between the sexes. Eleven case-patients had associated immunocompromise. Eleven were reportedly stable or improved at follow-up, 6 died, and 3 had uncertain outcomes.

Discussion

M. lentiflavum disease can be difficult to diagnose, as the cases in this report exemplify. The clinical information we gathered was largely retrospective, which poses certain limitations; however, the case-patients 1 and 2 were current patients undergoing active treatment at the time of writing (June 2009). *M. lentiflavum* was isolated occasionally from patients colonized or undergoing treatment for MAC and in patients with *S. aureus* soft tissue infections. Certainly in some patients multiple NTM can grow at the same or different times, and *M. lentiflavum* may be no different in this respect. *M. lentiflavum* has been cultured from sputum containing MAC and from sputum containing *M. tuberculosis*, but these cases may represent colonization/contamination rather than infection (*23*,*28*). Concurrent isolation of *M. lentiflavum* and *S. aureus*, which probably represents contamination or colonization, has not been reported as far as we are aware. Co-infection of *S. aureus* and *M. tuberculosis* has been reported, possibly as superimposed staphylococcal infection in tuberculous tissue (*29*,*30*). Although no samples were taken for histology, cytologic examination of lymph node aspirate from the 2-year-old child with lymphadenitis is intriguing because the inflammatory cells were predominantly lymphocytes/macrophages with epithelioid histiocytes. Treatment

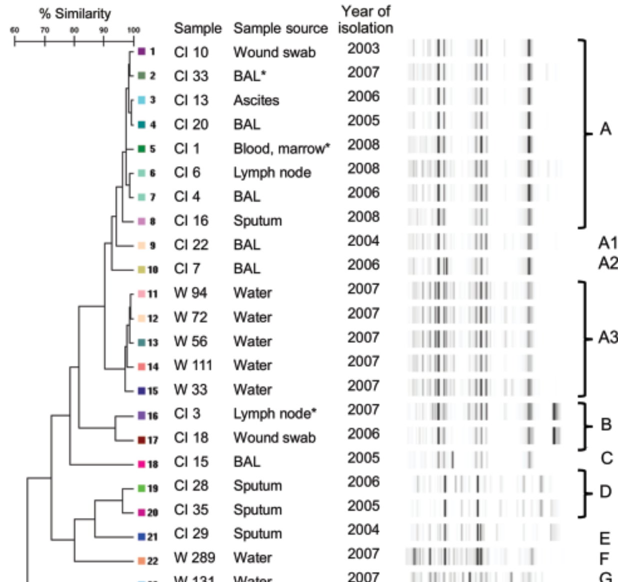

Figure 3. Dendrogram and virtual gel images representing rep-PCR fingerprint patterns of 16 human and 7 water isolates of *Mycobacterium lentiflavum*, Brisbane, Queensland, Australia, 2001–2008. CI, clinical isolate; W, potable water isolate; BAL, bronchoalveolar lavage. *Clinically significant isolate.

using flucloxacillin with or without drainage affected a complete cure in all cases.

M. lentiflavum is a rare isolate and an unusual cause of disease in humans. As with other NTM, it can be isolated from contaminated samples: clinical significance should be assessed before any treatment is considered (*2*). In 2005, of 488 patients with pulmonary NTM isolates in Queensland, only 26.6% were considered to have clinically significant disease (*31*). The proportion was higher for *M. intracellulare* (39.4%), *M. avium* (33.3%), and *M. kansasii* (52.6%) and much lower for species traditionally thought to be more likely contaminants, e.g., *M. gordonae* (11.1%). In our study, isolates for 4 (11%) of 36 were clinically significant, similar to published estimates of 10%–21% (*16*,*22*). This proportion may be an underestimate given that we could not determine clinical significance in 4 patients. Worldwide, *M. lentiflavum* may be underreported and incorrectly identified as other, more familiar species, especially if access to molecular identification is limited.

M. lentiflavum has been isolated from water distribution samples. Torvinen et al. isolated NTM from up to 80% of sites across Finland (*32*); *M. lentiflavum* was the second most common species (38% of sites). Laboratory isolation of *M. lentiflavum* from clinical specimens in Finland has increased independently of speciation methods, but details of patients with disease are lacking (*33*). In South Korea, Lee et al. found mycobacteria in 26% of 84 drinking water sites. Sixty-five percent of isolates were *M. lentiflavum* (*34*). In our study, mycobacteria were isolated from 70% of sites, but *M. lentiflavum* from only 6.3%. The difficulties in isolating mycobacteria from potable water are well recognized and relate to mycobacterial growth characteristics and the need for specimen decontamination to reduce bacterial and fungal overgrowth. Decontamination reduces mycobacterial yields; hence, the prevalence of mycobacteria in potable water samples is believed to substantially underestimate the true figure (*8*). Culture-based techniques may be less sensitive than direct PCR. However, detecting mycobacterial DNA does not necessarily prove the presence of viable organisms that are able to cause infection; detection of *M. lentiflavum* by culture-based methods is noteworthy with respect to human health. Case-patient 1 had long-term intravenous access, which may have allowed direct exposure to contaminated water through illicit drug administration. In this report, we have geographically associated culture-positive water samples and clinical disease.

DiversiLab strain typing showed that profiles A and A3 were most prevalent among clinical and water isolates and shared ≈90% similarity. The criteria for interpreting rep-PCR typing results have been established for some mycobacterial species. For example, Cangelosi et al. found high concordance between restriction fragment-length polymorphism and rep-PCR, reporting 93% similarity as the cutoff value for clustered *M. tuberculosis* isolates and 92% for *M. avium* (*6*). The analysis of *M. abscessus* by Zelazny et al., the largest study of rep-PCR in NTM, used rep-PCR to successfully cluster *M. abscessus* strains that were clonally related by PFGE analysis (*35*). Four of the water samples constituting profile A3 and 1 unrelated strain (profile G) came from sites that shared a groundwater source. These findings suggest a dominant environmental strain closely related (90%), but not identical, to strains found in human specimens and as a cause of human disease. The theory of dominant local environmental strains is supported by the finding of a different strain type from 2 patients living near each other but 450 km from Brisbane (profile D).

Profile A contained clinically significant and nonsignificant isolates. Profile B also contained a pair of highly similar isolates (94%) of which 1 was clinically significant. Although the residential addresses of these patients were 1,800 km apart, nothing is known about the duration of residence or travel or work habits of these case-patients. Thus, the infection may not have been acquired locally. Conclusions cannot be drawn about the pathogenicity of different strains; a larger study is required to address this question.

The finding of different, less common strain types (profiles E, F, G) confirms the validity of using automated rep-PCR (DiversiLab) as a tool for strain typing this species. Variation in *M. lentiflavum* strain type has been demonstrated. Buijtels et al. (*20*) reported 55 *M. lentiflavum* isolates from 149 specimens obtained from 38 patients at 1 hospital in Zambia. Illness of 2 patients definitely fulfilled ATS/IDSA criteria for significant disease. Because this species is a rare cause of disease, the authors performed molecular identification on a subset of 12 isolates to investigate the possibility of laboratory contamination. Six strain types were identified; the Zambian strains clearly differed from comparator Dutch strains. The finding of a dominant strain probably represented the local endemic strain, but laboratory or point-of-collection contamination could not be entirely excluded. Because isolates in our study came from multiple laboratories statewide at different times, contamination is unlikely to explain their presence in multiple clinical specimens.

The optimal treatment for *M. lentiflavum* disease is not established; a wide variety of regimens has been used in previous case series. Although evidence does not support the use of any specific regimen, we achieved symptomatic and radiologic improvement in case-patients 1 and 2 with rifampicin/ethambutol/clarithromycin at 12 months and isoniazid/ethambutol/clarithromycin at 18 months, respectively. More detailed reporting of treatment regimes and outcomes will help establish optimum therapy.

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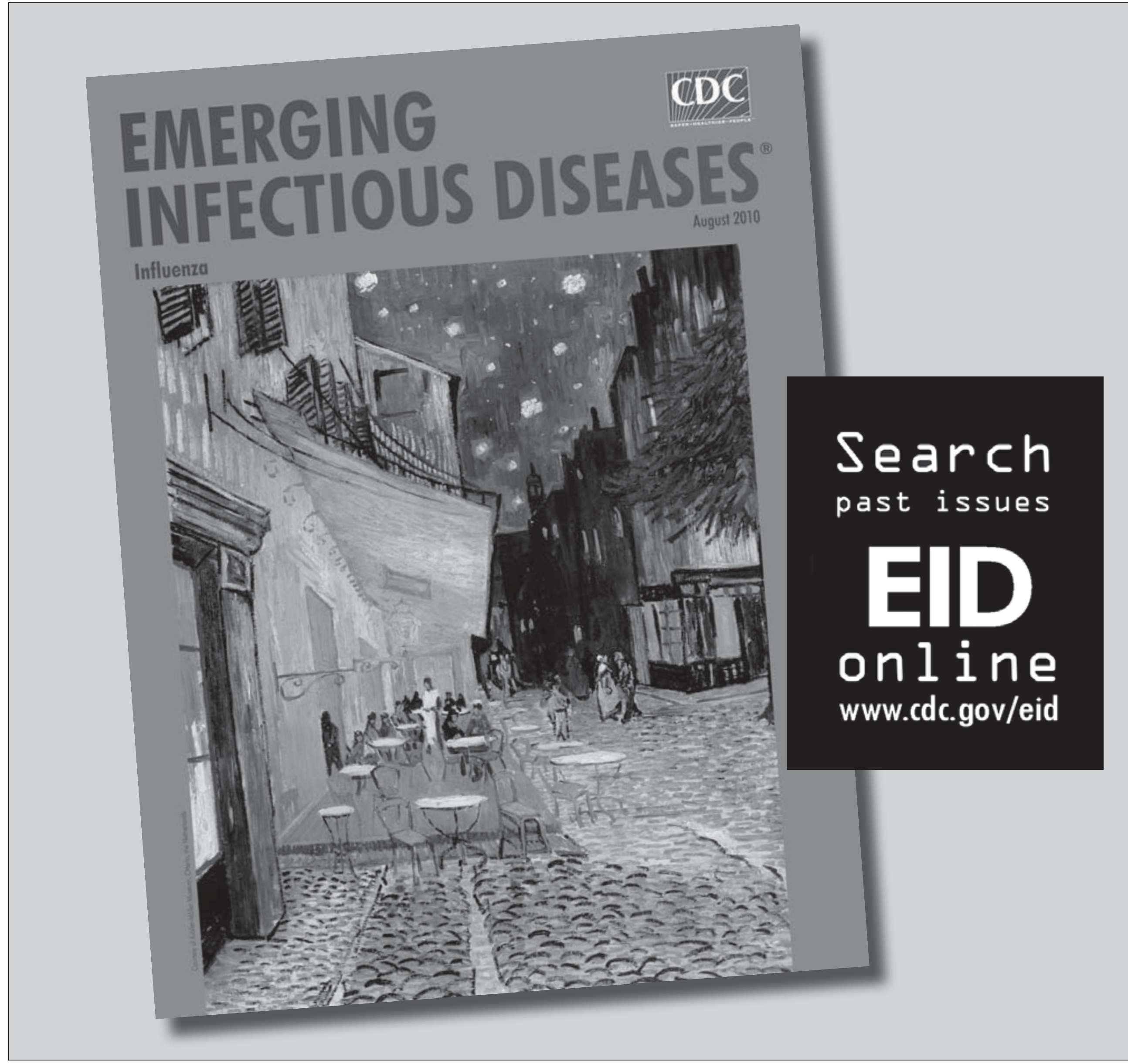

Swine Influenza Virus Antibodies in Humans, Western Europe, 2009

Nancy A. Gerloff, Jacques R. Kremer, Emilie Charpentier, Aurélie Sausy, Christophe M. Olinger, Pierre Weicherding, John Schuh, Kristien Van Reeth, and Claude P. Muller

Serologic studies for swine influenza viruses (SIVs) in humans with occupational exposure to swine have been reported from the Americas but not from Europe. We compared levels of neutralizing antibodies against 3 influenza viruses—pandemic (H1N1) 2009, an avian-like enzootic subtype H1N1 SIV, and a 2007–08 seasonal subtype H1N1—in 211 persons with swine contact and 224 matched controls in Luxembourg. Persons whose profession involved contact with swine had more neutralizing antibodies against SIV and pandemic (H1N1) 2009 virus than did the controls. Controls also had antibodies against these viruses although exposure to them was unlikely. Antibodies against SIV and pandemic (H1N1) 2009 virus correlated with each other but not with seasonal subtype H1N1 virus. Sequential exposure to variants of seasonal influenza (H1N1) viruses may have increased chances for serologic cross-reactivity with antigenically distinct viruses. Further studies are needed to determine the extent to which serologic responses correlate with infection.

Pandemic (H1N1) 2009 influenza virus resulted from genetic reassortment between at least 2 swine influenza viruses (SIVs) (*1*). Hemagglutinin (HA) of this novel subtype H1N1 virus is similar to that of classical swine influenza virus and the triple reassortant subtype H1N1 viruses that are endemic in swine populations in North America. At the time of its detection in humans, pandemic (H1N1) 2009 virus had never been detected in swine populations anywhere, but it is believed to have circulated undetected in regions with little or no surveillance for influenza viruses in swine. Because this virus has not been reported by the European Surveillance Network for Influenza in Pigs (www.esnip.ugent.be) since the network's inception in 2001, it was most likely absent in swine in western Europe. By the end of 2009, pandemic (H1N1) 2009 virus infection of swine had been reported in Norway (*2*); sporadic cases have been reported in a few other European countries (e.g., Germany, Italy, Denmark) (*3*). The swine were probably infected by contact with infected humans, whereas transmission from swine to humans has not yet been documented. Pandemic (H1N1) 2009 virus is the first swine-origin virus that is readily transmitted between humans (*4*).

Human infections with SIVs are rare. During 1958–2005, only 50 cases of zoonotic infections were reported; most were in persons who had contact with swine (*5*). Limited secondary transmission to close contacts has been reported but appears to be rare, and to our knowledge, sustained human-to-human transmission of enzootic SIVs has never been noted (*6*). Some serologic studies suggest that persons who work with swine are at increased risk for zoonotic infection with SIVs (*7–12*).

The predominant subtype H1N1 SIVs in Europe were introduced from wild ducks to swine in 1979 and have an entirely avian-derived genome (*13–15*). These viruses are designated as avian-like viruses and are antigenically distinct from subtype H1N1 SIVs in North America and from pandemic (H1N1) 2009 virus. Few cases of human infection with these avian-like swine subtype H1N1 viruses have been reported; chains of transmission have not been found (*5*,*9*,*15*), and no serologic studies have provided indirect evidence of transmission of SIVs to humans in Europe (*15*).

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Studies in the United States, United Kingdom, and Finland found antibodies against pandemic (H1N1) 2009 virus in elderly persons (*16–18*). These antibodies can be explained by antigenic evolution of seasonal human influenza (H1N1) viruses that are derived from the 1918 pandemic virus (such as the classical swine influenza [H1N1] virus) but have undergone greater antigenic drift than the swine virus (*19*). Antigenically, the influenza (H1N1) viruses that circulated among humans before the 1950s are probably more closely related to the classical swine virus and thus to the pandemic (H1N1) 2009 virus than to contemporary human subtype H1N1 viruses. We investigated whether persons whose professions involve contact with swine (swine workers [SWs]) have neutralizing antibodies against 3 influenza viruses: pandemic (H1N1) 2009 virus, a European avian-like subtype H1N1 SIV, and a 2007–08 seasonal influenza subtype H1N1 (seasonal influenza) virus.

Methods

Study Population

During July 20–28, 2009, blood was collected from 211 healthy persons with past or present professional contact with swine. All participants gave informed consent and completed a questionnaire about the nature of their swine contacts (occupation, duration, frequency), influenza vaccination, and influenza infection history. No participant reported having been infected with pandemic (H1N1) 2009 virus. A total of 224 control serum samples were obtained from the serum bank of the Laboratoires Reunis, Junglinster, Luxembourg. The samples, from the general population of Luxembourg, had been submitted in December 2008 for routine serologic testing. Because of ethical constraints, no further information was gathered from controls. The study was approved by the National Ethical Committee for Research in Humans.

Virus Neutralization Assay

According to recommended World Health Organization protocols (*20*), serum samples were tested by virus neutralization assay against an influenza A (H1N1) virus strain isolated from a patient in Luxembourg in July 2009 (A/Luxembourg/43/2009). Complete genome analyses revealed that the sequence was almost identical to the prototype vaccine virus (A/California/7/2009) and represented a typical North American/European pandemic (H1N1) 2009 virus (*4*). Nucleotide sequences are available from GenBank (accession nos. FN423708–15). A/swine/Belgium/1/98 is representative of the avian-like subtype H1N1 SIVs that are enzootic in swine populations of western Europe (*21*). Both viruses have an antigenically distinct H1 and ≈72% aa identity in the HA1 region (93% and 98% aa identity in neuraminidase [NA] and matrix [M] proteins) (*22*). A representative of the 2007–08 seasonal influenza virus was included in the assay and had 73% and 74% identity in HA1 proteins compared with pandemic (H1N1) 2009 virus and SIV (A/Luxembourg/572/2008 HA gene, accession no. FR716024).

Positive control serum was collected from 5 patients ≥5 weeks after recovery from a laboratory-confirmed infection with pandemic (H1N1) 2009 virus and from a previously unexposed pig 4 weeks after it had been experimentally infected with A/swine/Belgium/1/98 (H1N1) (*21*). Before the assay was conducted, all samples were heated to 56°C for 30 min to inactivate complement and unspecific inhibitors. Titers were reported as the reciprocal of the highest dilution of serum that completely neutralized virus growth. Samples were first screened in duplicate with a 1:10 dilution. All samples that showed virus neutralization in ≥1 well were further titrated in quadruplicate up to a dilution of at least 1:320. Control samples positive for both viruses were included in all assays.

Statistical Analyses

Geometric mean titers (GMTs) were calculated for each person from quadruplicate serum samples. All negative samples were given an arbitrary GMT of 5. GMTs were compared by using the nonparametric Wilcoxon rank-sum test. To examine bivariate risk factors associated with antibody prevalence, we dichotomized GMTs of all positive samples for different cutoff points (≥10 to ≥80) and analyzed them by χ^2 test and, for low proportions, by *z*-test. The distribution of antibody levels was checked for associations with multiple risk factors by using proportional odds modeling (*23,24*). Statistical analyses were performed by using SigmaStat version 3.1 (San Jose, CA, USA) and SPSS version 18 (Chicago, IL, USA).

Results

Study Population

Mean age of the 211 SWs was 48.2 years (range 18–94 years); 67.8% were male (Table 1). Most (84.8%) SWs reported having worked daily in close contact with swine (distance <1 m, 83%) for >10 years (73.5%). Among the SWs, 133 were involved in pig breeding, fattening, or general pig farming; 51 were slaughterhouse workers; 12 were veterinarians; 13 were butchers; and 2 were hunters. The 224 controls were matched with SWs by age and sex (Table 1).

Antibodies against Pandemic (H1N1) 2009 Virus

GMTs of antibodies against pandemic (H1N1) 2009 virus (Table 2) were significantly higher for SWs than for controls (p = 0.004). Table 3 shows that 2× more SWs

Table 1. Characteristics of persons tested for 3 influenza viruses, Luxembourg, 2008–2009*

Characteristic	Swine workers, no. (%),† n = 211	Controls, no. (%),‡ n = 224
Sex		
M	143 (67.8)	151 (67.4)
F	68 (32.2)	73 (32.6)
Age group, y§		
18–40	69 (32.7)	80 (35.7)
41–50	59 (28)	58 (25.9)
51–60	39 (18.5)	41 (18.3)
61–94	44 (20.9)	45 (20.1)
Profession		
Farmer	133	NA
Slaughterhouse worker	51	NA
Other¶	27	NA
Years worked with swine		
<1	4 (1.9)	NA
1–4	26 (12.3)	NA
5–10	26 (12.3)	NA
>10	155 (73.5)	NA
Unknown	0	224
Frequency of swine contact		
Rarely	3 (1.4)	NA
Monthly	2 (0.9)	NA
Weekly	25 (11.8)	NA
Daily	179 (84.8)	NA
Unknown	2 (0.9)	NA
Frequency of close contact (<1 m) with swine		
Never	1 (0.5)	NA
Rarely	3 (1.4)	NA
Occasionally	10 (4.7)	NA
Often	22 (10.4)	NA
Always	175 (82.9)	NA
Self-reported influenza vaccine in past 5 y		
No/unsure	155 (73.5)	NA
Yes	56 (26.5)	NA
Self-reported infection with seasonal influenza		
No	145 (68.7)	NA
Yes	57 (27.0)	NA
Exposure to swine		
Only until 1997	26	NA
Only until 2007	59	NA
Until time of collection	152	NA

*NA, not available.
†Sampled in July 2009.
‡Sampled in December 2008.
§Mean (median) age 48.2 (48) years for swine workers, 47.6 (47.2) years for controls.
¶Veterinarian, butcher, hunter.

than controls had neutralizing antibodies against pandemic (H1N1) 2009 virus for the lowest cutoff value (p = 0.001). This ratio slightly increased with rising cutoff values and remained significant to a cutoff ≥160 (Table 3). In all age groups, ≈2× more SWs than controls had antibodies against pandemic (H1N1) 2009 virus (cutoff ≥10), except for persons >60 years of age (Table 4). For SWs and controls >60 years of age, GMTs for pandemic (H1N1) 2009 virus were similar (p = 0.897; Table 2). GMTs were significantly higher for younger than for older (>60 years) SWs (but not controls) (Table 2). Among SWs, antibodies against pandemic (H1N1) 2009 virus tended to decrease with age for all cutoff values; among controls, the same was observed for cutoffs ≥10 to ≥40. Thus, younger SWs more often had higher levels of antibodies against pandemic (H1N1) 2009 virus than did controls and older SWs. The difference between SWs and controls disappeared in older age groups and was weaker when older and younger controls were compared.

Antibodies against SIV

Similar to findings for pandemic (H1N1) 2009 virus, GMTs for SIV were higher among SWs than controls; however, the difference was not significant (Table 2; p = 0.168). More SWs than controls had positive SIV titers regardless of the cutoff (Table 3). These differences were significant for cutoffs ≥20 to ≥160 and increased with higher cutoffs (Table 3). Comparable to findings for pandemic (H1N1) 2009 virus, for age groups up to 60 years antibodies against SIV were found in 1.2–2× more SWs than controls (cutoff ≥10; Table 4); GMTs were significantly higher among SWs than controls in this age group (Table 2; p = 0.028). Seroprevalences and GMTs were similar for persons >60 years of age from each group (Tables 2, 4).

In contrast to findings for pandemic (H1N1) 2009 virus, the highest proportion of seropositive persons was found in older age groups, SWs >50 and controls >60 years (Table 4). GMTs were significantly higher among older (>60 years) than younger controls (p = <0.001) but differed little among SWs (Table 2; p = 0.293).

Thus, antibody titers for SIV were found more often and were higher among SWs than controls. In contrast to findings for pandemic (H1N1) 2009 virus, titers for SIV were found more often and were higher for older than younger controls; for SWs, titers were found more often among older persons but values were similar.

Antibodies against Pandemic (H1N1) 2009 Virus and SIV

Among SWs, for all cutoff values seroprevalence was higher for SIV than for pandemic (H1N1) 2009 virus. The same was found for controls but only for lower titers (≥10 and ≥20; Table 3). The differences between antibody positivity for each of the 2 viruses increased with age among SWs and controls (Table 4). Comparing seroprevalences for pandemic (H1N1) 2009 virus to those for SIV, differences were significant only for SWs >60 years (p = 0.002). Also, significantly more controls of the same age group (>60 years) had antibodies against SIV (62.2%) than against pandemic (H1N1) 2009 virus (6.7%, p<0.001; Table 4). The proportion of older (>60 years) SIV-seropositive controls (62.2%) differed significantly from the proportion

Table 2. Geometric mean titers for 3 influenza viruses in swine workers and controls, Luxembourg, 2008–2009*

Virus (strain) and participant age, y	Study sample, % (95% CI)		p value†
	Swine workers, n = 211	Controls, n = 224	
Pandemic (H1N1) 2009 (A/Luxembourg/43/2009)			
All	8.7 (7.5–10)	6.1 (5.6–6.6)	**0.004**
<60	9.2 (7.6–11.1)‡	6 (5.5–6.7)	**<0.05**
≥60	5.6 (4.5–6.9)	5.4 (4.6–6.4)	0.897
Avian-like SIV (H1N1) (A/swine/Belgium/1/98)			
All	10.3 (8.8–12)	7.7 (6.9–8.5)	0.168
<60	9.8 (8.1–11.8)	6.4 (5.8–7)§	**<0.05**
≥60	11.2 (8–15.5)	13.6 (9.9–18.5)	0.170
Seasonal influenza (H1N1) (A/Luxembourg/572/2008)¶			
All	23.2 (20.3–26.4)	13.9 (12.1–15.9)	**<0.001**
<60	21.3 (18.3–24.7)	12.4 (10.7–14.4)#	**<0.001**
≥60	30.6 (22.7–41.1)	20.1 (15.3–28.7)	0.083

*%, no. persons/total no. persons in age groups with geometric mean titer cutoff ≥10. SIV, swine influenza virus.
†p value <0.05 for significance were calculated by using the Wilcoxon rank-sum test. **Boldface** indicates significance (p<0.05).
‡p<0.05, compared with swine contacts of the age group >60 y against pandemic (H1N1) 2009 virus.
§p<0.001, compared with controls of the age group >60 y against avian-like SIV.
¶Data for 210 swine workers, 221 controls.
#p = 0.001, compared with controls of the age group >60 y against seasonal influenza (H1N1).

of younger (<60 years) SIV-seropositive controls (17.3%; p<0.001).

Thus, for both groups, more persons had antibodies against SIV than against pandemic (H1N1) 2009 virus, and differences in positivity decreased with increasing titers. Antibodies against SIV were more common among older persons, and antibodies against pandemic (H1N1) 2009 virus were more common among younger persons.

Antibodies against SIV and Pandemic (H1N1) 2009 Virus

Antibody titers of convalescent-phase serum samples from patients with pandemic (H1N1) 2009 virus were 16× higher for pandemic (H1N1) 2009 virus than for SIV (GMTs 226.2 vs. 13.5, respectively), indicating low cross-reactivity between these viruses. Similarly, in a pig serum sample, GMT for SIV (>1,280) was 128× lower than that for pandemic (H1N1) influenza (*8*).

Table 3. Neutralizing antibody reactivity against 3 influenza viruses in swine workers and controls, Luxembourg, 2008–2009*

Virus (strain) and cutoff value	Swine workers, no. (%; 95% CI), n = 211	Controls, no. (%; 95% CI), n = 224	p value
Pandemic (H1N1) 2009 (A/Luxembourg/43/2009)			
≥10	46 (21.8; 16.8–27.9)†	23 (10.3); 6.9–14.9)‡	**0.001§**
≥20	37 (17.5; 13–23.2)†	16 (7.1; 4.4–11.3)‡	**0.001§**
≥40	31 (14.7; 10.6–20.1)	12 (5.4; 3.1–9.1)	**0.002§**
≥80	14 (6.6; 4–10.8)	4 (1.8; 0.7–4.5)	**0.02§**
≥160	6 (2.8; 1.3–6.06)	0 (0; 0–1.2)	**0.033¶**
≥320	5 (2.4; 1–5.4)	0 (0; 0–1.2)	0.061¶
Avian-like SIV (H1N1) (A/swine/Belgium/1/98)			
≥10	66 (31.3) 25.4–37.8)	59 (26.3; 21–32.5)	0.289§
≥20	57 (27; 21.5–33.4)	38 (17; 12.6–22.4)	**0.015§**
≥40	39 (18.5; 13.8–24.3)	12 (5.4; 3.1–9.1)	**<0.001§**
≥80	21 (10; 6.6–14.7)	4 (1.8; 0.7–4.5)	**<0.001§**
≥160	9 (4.3; 2.3–7.9)	1 (0.4; 0.1–2.5)	**0.019¶**
≥320	4 (1.9; 0.7–4.8)	1 (0.4; 0.1–2.5)	0.331¶
Seasonal influenza (H1N1) (A/Luxembourg/572/2008)#			
≥10	183 (87.1; 83–91.9)	132 (59.7; 53.2–66)	**<0.001§**
≥20	125 (59.5; 52.8–65.9)	76 (34.4; 28.4–40.9)	**<0.001§**
≥40	61 (29; 23.3–35.5)	39 (17.6; 13.2–23.2)	**0.007§**
≥80	21 (10; 6.6–14.9)	17 (7.7; 4.8–12)	0.500§
≥160	14 (6.7; 3.9–11)	7 (3.2; 1.4–6.5)	0.144§
≥320	11 (5.2; 2.9–9.2)	4 (1.8; 0.5–4.7)	0.093§

*Values are no. persons with antibodies. CI, confidence interval; SIV, swine influenza virus. **Boldface** indicates significance (p<0.05).
†p<0.05 when compared with swine workers against avian-like SIV (H1N1) of the same titer cutoff.
‡p<0.003 when compared with swine workers against avian-like SIV (H1N1) of the same titer cutoff.
§χ^2 test on 2-way table.
¶z-test.
#Data for 210 swine workers, 221 controls.

Table 4. Neutralizing antibody reactivity ≥10 for 3 influenza viruses in swine workers and controls, Luxembourg, 2008–2009*

Participant age in 2009, y	Pandemic (H1N1) 2009 (A/Luxembourg/43/2009)		Avian-like SIV (H1N1) (A/swine/Belgium/1/98)		Seasonal influenza (H1N1) (A/Luxembourg/572/2008)	
	Swine workers	Controls	Swine workers	Controls	Swine workers	Controls
≤40	22/69 (31.9; 22.1–43.6)†	12/80 (15; 8.8–24.4)	19/69 (27.5; 18.4–39.0)	15/80 (18.8; 11.7–28.7)	58/68 (85.3; 6.9–93.7)	50/78 (64.1; 53.5–74.8)
41–50	10/59 (16.9; 9.5–28.5)	5/58 (8.6; 3.7–18.6)	11/59 (18.6; 10.7–30.4)	8/58 (13.8; 7.2–24.9)	46/59 (78; 67.4–88.6)	29/57 (50.9; 37.9–63.9)
51–60	9/39 (15.3; 12.7–38.3)	3/41 (7.3; 2.5–19.4)	17/39 (43.6; 29.3–59.0)‡	8/41 (19.5; 10.2–34.0)	37/39 (94.9; 88.0–101.8)§	19/41 (46.3; 31.1–61.6)
>60	5/44 (8.6; 5.0–24.0)¶	3/45 (6.7; 2.3–17.9)#	19/44 (43.2; 29.7–57.8)	28/45 (62.2; 47.6–74.9)	42/44 (95.5; 89.3–101.6)**	34/45 (75.6; 63.0–88.1)
18–94 (total)	46/211 (21.8; 16.8–27.9)††	23/224 (10.3; 6.9–14.9)‡‡	66/211 (31.3; 25.4–37.8)	59/224 (26.3; 21.0–32.5)	183/210 (87.1; 83.0–91.9)	132/221 (59.7; 53.2–66.0)

*Values are no. persons/total no. persons in age groups with antibody reactivity ≥10 (%; 95% confidence interval). p values <0.05 cutoff for significance were calculated by using the χ^2 test. SIV, swine influenza virus.
†p = 0.012, compared with controls of the same age group against pandemic (H1N1) 2009.
‡p = 0.037, compared with controls of the same age group against avian-like SIV (H1N1).
§p<0.001, compared with controls of the same age group against seasonal influenza (H1N1).
¶p = 0.002, compared with swine workers of the same age group against avian-like SIV (H1N1).
#p<0.001, compared with controls of the same age group against avian-like SIV (H1N1).
**p<0.05, compared with controls of the same age group against seasonal influenza (H1N1).
††p<0.05, compared with swine workers against avian-like SIV (H1N1).
‡‡p<0.001, compared with controls against avian-like SIV (H1N1).

Among 66 SIV-positive serum samples from SWs, only 28 were also positive for pandemic (H1N1) 2009 virus (GMT cutoff ≥10). GMTs of at least single positive samples correlated significantly with each other (R^2 = 0.5, correlation coefficient [CC] = 0.4, p<0.001; Figure, panel C); and GMTs for SIV were significantly higher than corresponding GMTs for pandemic (H1N1) 2009 virus (48, 95% CI 38.4–60.1, and 16.3, 95% CI 11.3–22.8, respectively; p<0.001). To the contrary, among SIV-positive controls GMTs for SIV did not correlate with GMTs for pandemic (H1N1) 2009 virus (R^2<0.01, CC = 0.322; Figure, panel D).

Among SWs, being SIV positive increased the odds of being positive for pandemic (H1N1) 2009 virus by 2.4× (odds ratio [OR] 95% CI 1.3–4.3). Among controls, these chances were increased by 6× (OR 95% CI 2.9–12.6).

Seasonal Influenza Virus Compared with Pandemic (H1N1) 2009 Virus and SIV

GMTs for seasonal influenza virus were significantly higher among SWs than controls (Table 2), and significantly more SWs than controls had antibodies against seasonal influenza virus, at least for titers ≈10 to 40 (Table 3). Among all age groups, more SWs than controls had antibodies against seasonal influenza (Table 4). GMTs among controls >60 years of age were significantly higher than those among younger controls (Table 2). Significantly more SWs and controls had antibodies against seasonal influenza virus than against pandemic (H1N1) 2009 virus and SIV (Table 3).

All SWs with antibodies against pandemic (H1N1) 2009 virus also had antibodies against seasonal influenza virus with the following exceptions: 1) GMTs were significantly higher for pandemic (H1N1) 2009 virus (50.8, 95% CI 37.7–68.4) than for seasonal influenza viruses (31.5, 95% CI 26.6–37.4) (p = 0.001); 2) GMTs of at least single positive serum samples did not correlate (R^2<0.01, CC = 0.231; Figure, panel A); and 3) 17 of 21 samples with seasonal influenza virus titers ≥80 were negative for pandemic (H1N1) 2009 virus (cutoff <10). No correlation was found between GMTs of samples positive for seasonal influenza virus and SIV (R^2<0.01, CC = 0.339; Figure, panel B). These results may indicate no substantial cross-reactivity between antibodies against pandemic (H1N1) 2009 virus or SIV and at least a recent seasonal influenza virus.

Risk Factors

Odds of having antibodies against pandemic (H1N1) 2009 virus were 2.4× (95% CI 1.4–4.2) to 3.9 (95% CI 1.3–12) greater for SWs than for controls (cutoffs ≥10 to ≥160). Odds of having antibodies against SIV were 1.3× (95% CI 0.8–1.9) to 9.9 (95% CI 0.5–38.9) greater for SWs than for controls (cutoffs ≥10 to ≥80). Odds of being SIV positive were slightly higher for farmers (OR 2.3, 95% CI 1.1–5) than for slaughterhouse workers; odds of being positive for pandemic (H1N1) 2009 virus were only slightly higher for farmers than for slaughterhouse workers (OR 1.2, 95% CI 0.6–2.5). ORs for being positive for pandemic (H1N1) 2009 virus and for SIV were slightly higher for male SWs (1.7, 95% CI 0.8–3.5, and 1.1, 95% CI 0.6–2.3, respectively; cutoff ≥10). Among SWs, 26.5% self-reported receiving ≥1 dose of seasonal influenza vaccine during the past 5 years; among vaccinated SWs, the odds of having antibodies against pandemic (H1N1) 2009 virus (OR 1.3, 95% CI 0.6–2.6) as well as against SIV (OR 1.3, 95% CI 0.7–2.5; cutoff

≥10) were slightly higher than those for unvaccinated SWs. Odds of having antibodies against pandemic (H1N1) 2009 virus were slightly higher for SWs exposed to swine until the time of sampling (OR 1.5, 95% CI 0.7–3.3) in 2009 than for those who had no contact with swine after 2007. OR for having antibodies against SIV for SWs with pig contact until time of sampling was 0.5 (95% CI 0.2–1.1) compared with that for persons who had no contact after 1997. Thus, no significant associations were found between year of exposure and seroprevalence of antibodies against either virus.

Discussion

At the time of blood collection from SWs (late July 2009), pandemic (H1N1) 2009 had spread to all continents, but intensity was still low in Europe, especially in Luxembourg and its neighboring countries. The only countries in which infection rates increased were the United Kingdom, Ireland, and Spain (where sporadic outbreaks occurred) (*25*). In 2009, Luxembourg had an intensive active surveillance system for influenza-like illnesses. Follow-up for all patients with suspected cases included patient travel history, RNA extraction, and PCR to detect pandemic (H1N1) 2009 virus. All patients with confirmed

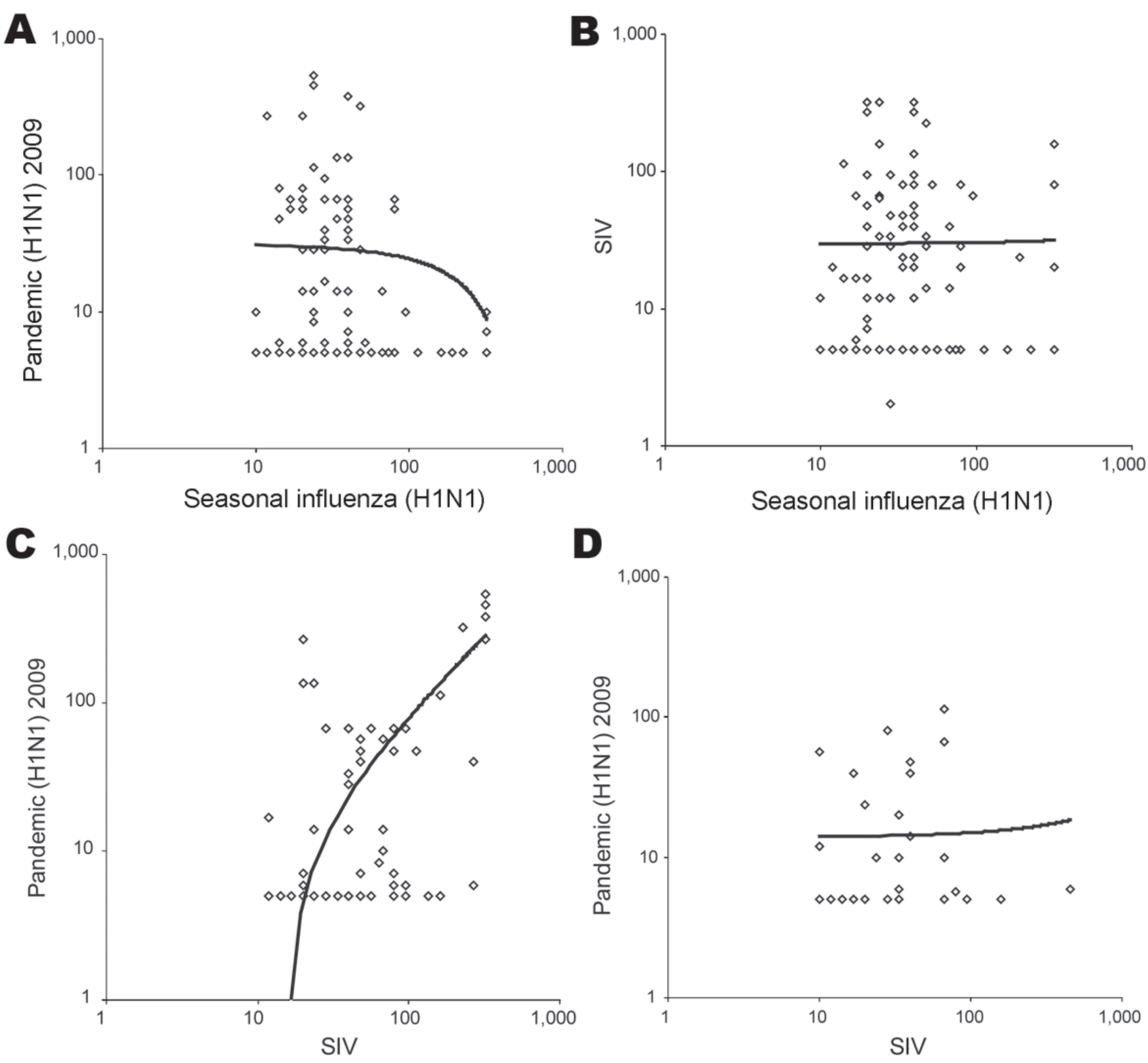

Figure. Geometric mean titers (≥10) of antibodies against pandemic (H1N1) 2009 virus, seasonal influenza (H1N1) virus, and swine influenza virus of swine workers (A, B, C) and controls (D). Each symbol represents titer of 1 person; only persons with positive results (≥10) for at least 1 of the 2 viruses of the panel are shown. Trend lines are shown; R^2 values were R^2>0.01 for panels A, B, and D and R^2 = 0.5 for panel C.

disease were monitored until at least early August. Patients and their contacts received prompt antiviral drug treatment, and home quarantine was recommended. In Luxembourg, ≈60 cases were reported and confirmed around the time that blood collection from SWs was ending. Until end of June 2009, almost all Luxembourg patients were epidemiologically unrelated, and the source of infection was not determined for one fifth (*26*). The first sustained transmissions were noted by mid September (J. Mossong, pers. comm.). The first cases of pandemic (H1N1) 2009 in swine on the European mainland were reported in January 2010 (*3*). Nevertheless, the difference in the time of blood collection from controls (December 2008) and from SWs (July 2009) is a limitation of our study.

The virus neutralization assay used measures neutralizing antibodies mainly against HA because antibodies were in the assay only during the virus entry phase (*20*). Nevertheless, we cannot exclude that residual antibodies against NA and M (93% and 98% aa identity between pandemic [H1N1] 2009 virus and SIV, respectively) may contribute to neutralization (*27*).

Because there is no correlate of protection for neutralizing antibodies or a definition of a positive titer measured by virus neutralization assay (*28*), we analyzed titers by using running cutoff values for positivity and compared GMTs. This analysis showed significantly higher prevalence of neutralizing antibodies against pandemic (H1N1) 2009 virus in SWs than in controls, and seropositivity decreased with age. Younger (<60 years) SWs had higher titers, and 2× more SWs than age-matched controls had neutralizing antibodies against pandemic (H1N1) 2009.

No evidence indicates that pandemic (H1N1) 2009 virus was present in swine in Europe in or before July 2009. Reactivity with pandemic (H1N1) 2009 virus correlated best with antibodies against SIV. Although this correlation was highly significant among SWs with relatively high titers for SIV, no such correlation was found among controls, in whom antibody levels against SIV were low. We speculate that the difference between the cohorts may reflect cross-reactive antibodies to another influenza virus more similar to SIV (with or without a minor contribution of antibodies against seasonal influenza) in SWs, in contrast to low, mainly cross-reacting seasonal influenza virus antibodies in controls. Serologic cross-reaction between SIV and pandemic (H1N1) 2009 virus in pigs was recently reported (*22*). Our results also showed that reactivity with pandemic (H1N1) 2009 (or SIV) in either cohort cannot be explained by cross-reactivity with a recent seasonal influenza virus used in this study. Nevertheless, because more SWs than controls were exposed to seasonal influenza virus, we cannot exclude the possibility that antibodies to pandemic (H1N1) 2009 virus or to SIV in the SWs may be caused by a more complex history of exposure to seasonal influenza virus of subtype H1 or to subclinical infections with pandemic (H1N1) 2009 virus during the first months of the pandemic.

Our finding of low levels of neutralizing antibodies against pandemic (H1N1) 2009 in controls (general population) is in agreement with findings of previous studies (*29*). Our findings that titers were less common but higher for older controls contrast with reports from the United Kingdom and Finland (*16,17*) but agree with findings of 2 studies in China, where elderly persons (≥60 years) had few or no neutralizing antibodies against this virus (*30,31*).

Our study also showed significantly higher prevalence of neutralizing antibodies against SIV in SWs than in the controls at cutoffs ≥20 to ≥160, but differences in GMTs were not significant. Similar serologic studies in humans in the United States showed markedly elevated antibody titers for North American SIVs of subtype H1N1 and H1N2 in SWs compared with controls (*5*,*8*,*10*,*11*,*32*,*33*). These studies used hemagglutination inhibition instead of virus neutralization assays and reported ORs for increased serologic responses instead of seroprevalence rates. The reported ORs, however, seem to be higher than those in our study (*8*,*32*,*33*) and could be partially explained by exclusion of persons with swine exposure in the US control groups.

Most persons undergo sequential infections with multiple antigenic variants of human influenza subtype H1N1 and H3N2 viruses throughout their lives. Such infections strongly increase the odds for serologic cross-reactions with antigenically distinct H1 viruses, as documented in experimental studies with pigs (*22*), and may explain why older persons in the general population have higher antibody titers to SIV than their younger counterparts. Both older and younger controls are unlikely to have been infected with SIV, but older persons have been exposed to a wider variety of human seasonal influenza viruses. This exposure is also reflected by a significant difference in GMTs for recent seasonal influenza virus in older than younger controls. In Luxembourg, elderly persons may have had contact with swine because during 1920–1947 in Luxembourg, 50%–22% of all households kept ≥5 pigs, but before 1979, there was no apparently substantial swine influenza activity in this part of Europe (*14*). Apart from antibodies to SIV, a few controls also had antibodies to pandemic (H1N1) 2009 virus, but these did not correlate with each other, suggesting a different cross-reactivity pattern than that for SWs. These findings show that in the absence of paired serum samples, presence of neutralizing antibodies to a given influenza virus does not necessarily reflect infection with that virus. Elevated antibody titers to SIV in part of the SWs may have resulted

from exposure to the virus, but further studies are required to determine all possible causes.

In conclusion, titers of antibodies against pandemic (H1N1) 2009 virus and against an avian-like subtype H1N1 influenza virus were found more frequently and were higher for SWs than for controls. These titers cannot be explained by cross-reactivity with antibodies from recent seasonal influenza viruses. Neutralizing antibodies to both subtype H1N1 viruses showed some degree of correlation.

Further studies are needed to determine incidence of zoonotic SIV infections and the extent to which serologic responses correlate with infection. Neutralizing antibodies should confer at least partial protection against infection, reducing the risk that the avian-like subtype H1N1 SIV will cause major outbreaks of disease in humans.

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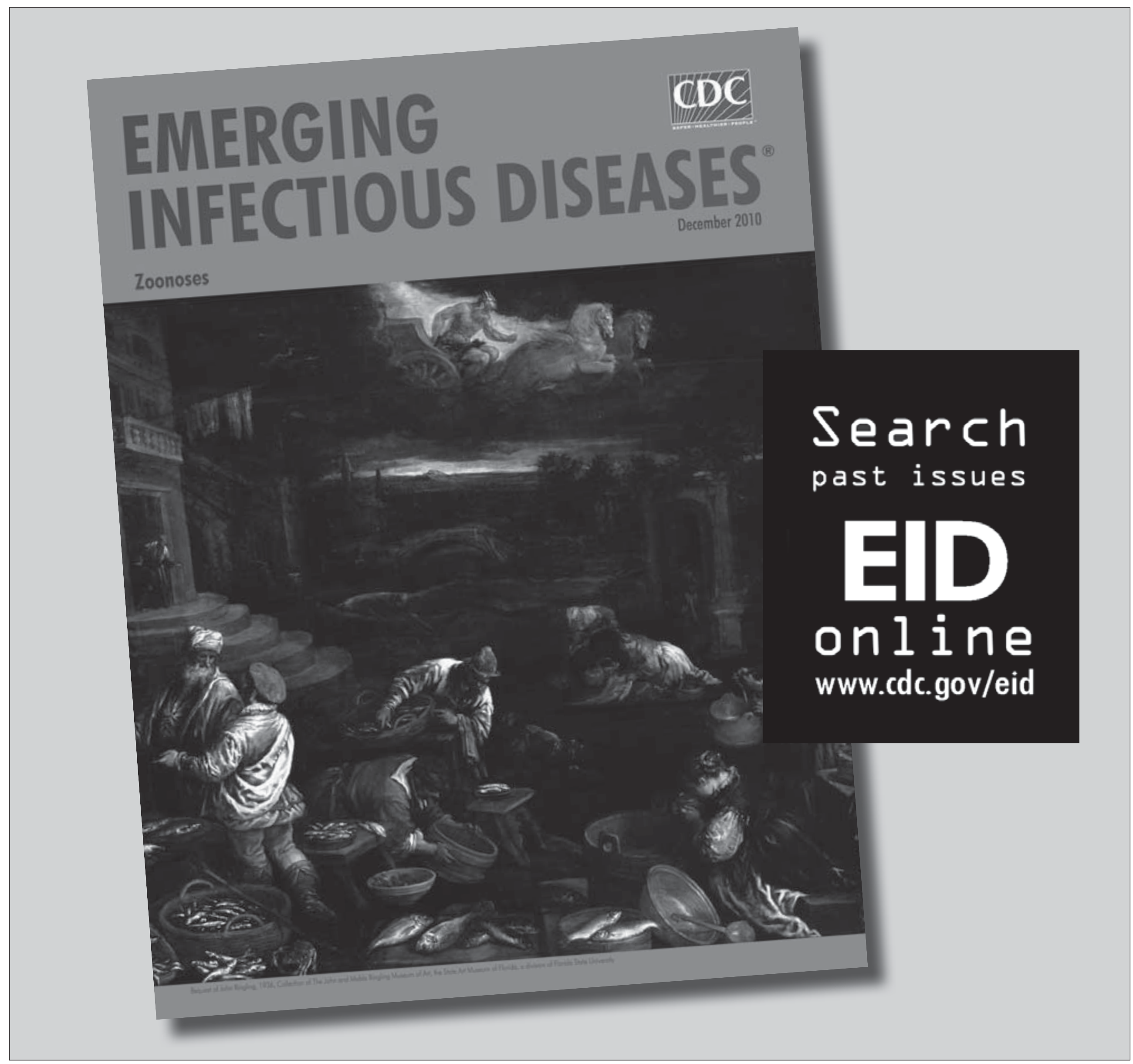

An Integrated Approach to Identifying International Foodborne Norovirus Outbreaks[1]

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International foodborne norovirus outbreaks can be difficult to recognize when using standard outbreak investigation methods. In a novel approach, we provide stepwise selection criteria to identify clusters of outbreaks that may involve an internationally distributed common foodborne source. After computerized linking of epidemiologic data to aligned sequences, we retrospectively identified 100 individually reported outbreaks that potentially represented 14 international common source events in Europe during 1999–2008. Analysis of capsid sequences of outbreak strains (n = 1,456), showed that ≈7% of outbreaks reported to the Food-Borne Viruses in Europe database were part of an international event (range 2%–9%), compared with 0.4% identified through standard epidemiologic investigations. Our findings point to a critical gap in surveillance and suggest that international collaboration could have increased the number of recognized international foodborne outbreaks. Real-time exchange of combined epidemiologic and molecular data is needed to validate our findings through timely trace-backs of clustered outbreaks.

Noroviruses are the most prevalent causative agents of acute viral gastroenteritis in the community (*1*–*4*). Currently, 5 norovirus genogroups have been described and subdivided into at least 40 genotypes (*5*,*6*), but in recent years, most clinical effects have been caused by viruses from a single genotype in genogroup II, GII.4 (*7*–*10*). The symptoms of norovirus disease are usually mild and self-limiting, but there is some evidence the disease can contribute to proportion of deaths (*11*,*12*). Infection occurs by way of the gastrointestinal tract after contact with infected persons, after ingestion of contaminated food or aerosols, or through environmental contamination (*13*,*14*).

Because the different modes of transmission call for quite distinct control measures, it is important to assess which proportion of disease can be attributed to which mode of transmission. However, this question is difficult to answer. Due to the high rate and rapidity of secondary spread of norovirus infection following a foodborne introduction, outbreaks initially linked to a food source may appear to be person-to-person (PTP) outbreaks by the time they are recognized. Even if a foodborne source is suspected, confirmation of the source is complicated. Virus detection in food commodities is possible but hampered by such factors as low levels of norovirus in food, food matrix complexity, genetic variability of norovirus (*15*), the absence of an efficient cell culture system to propagate human noroviruses (*16*), and the unavailability of leftover food for pathogen detection.

Given the globalization of the food market, diffuse international outbreaks are likely (*17*,*18*). For public health officials, these may seem to be regular PTP outbreaks because infection of 1 or a few persons with viruses through food consumption will go unnoticed unless secondary spread occurs or the contaminated food is consumed by multiple persons, which may trigger an investigation to identify a source. However, identification of international links is complicated. Viruses remain

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[2]Members of the Food-Borne Viruses in Europe Network who contributed to this study are listed at the end of this article.

infectious in frozen ready-to-eat products over prolonged periods, and linked outbreaks are likely to be separated in time (*19*). Other problems are virus mutation rate, which results in nonidentical strains from a common source (*20*); sewage contamination with multiple nonsimilar strains during production of shellfish or crops (*21*); underreporting of cases (*22*,*23*); and incompleteness of outbreak reports (*24*,*25*). Other complicating factors include the unknown background level of viruses in foods, the environment, or asymptomatic shedders. Clearly, methods combining molecular and basic epidemiologic criteria are needed to assist public health efforts to identify international foodborne outbreaks.

For this reason, we performed a retrospective analysis of norovirus outbreak surveillance data collected since 1999 by Food-Borne Viruses in Europe (FBVE), a combined laboratory and epidemiology network (*6*). Although the name FBVE suggests a foodborne focus, the network actually investigates outbreaks of viral gastroenteritis with all modes of transmission. It seeks to obtain a comprehensive overview of viral activity in the community and to enable capture of foodborne norovirus outbreaks that have evaded recognition. Strain sequences from outbreaks linked to a common source are expected to be more similar than strains from outbreaks with a different source (*26*). We sought to quantify strain variability within and among molecular sequence clusters of multiple outbreaks to identify outbreaks with probable links to other outbreaks. Our goal was to retrospectively identify potential common-source events not detected by routine investigations and also to provide criteria that may assist in detecting such events.

Methods

Definitions

A norovirus outbreak was reported to FBVE when it included a minimum of 2 patients in the same area within 2 days who had ≥2 instances of vomiting and/or watery diarrhea within a 24-hour period (*6*,*27*). A gastroenteritis outbreak was ascribed to norovirus on the basis of compatible descriptive epidemiology and laboratory confirmation in at least 2 of 5 feces samples tested (*28*). An outbreak strain was defined as a sequenced norovirus strain considered representative of an outbreak (found preferably in ≥2 samples from patients in the same outbreak). If dissimilar sequences were detected, multiple strains were considered representative. A genotype is a group of closely related noroviruses, i.e., showing >80% similarity in the complete capsid amino acid sequence. Genotypes can be assigned based on shorter sequences if a full capsid was previously identified and sequenced for comparison (*29*). In this report, a cluster refers to a molecular cluster of multiple outbreaks, not an epidemiologic cluster of patients in 1 outbreak. A cluster of similar sequences is a group of outbreak strain sequences in the same genotype that show a minimal number of mutations within the region of overlap; the exact number of mutations depends on the sequence length in the region and the cutoff value used to define similarity. A cluster of identical sequences is a group of outbreak strain sequences with the highest possible similarity (100%). According to reporting standards of the FBVE network (*24*,*30*), the suspected mode of transmission during an outbreak was considered foodborne when the infection was related to consumption of food contaminated during its production or processing; food handler–borne (FHB) when infection related to food prepared by an infected food handler; person-borne when it related to direct contact with infected persons; and unknown (UN) when no mode could be identified.

Selection of Strains Representing Outbreaks

From January 1999 through November 2008, the FBVE network collected molecular information on a total of 5,499 norovirus outbreaks in Denmark, Finland, France, England and Wales, Germany, Hungary, Ireland, Italy, the Netherlands, Norway, Slovenia, Spain, and Sweden (*24*,*30*). Strengths and limitations of the FBVE data collection have been described (*24*). FBVE data are reported aggregated at outbreak level. Consequently, throughout the analysis here described, a strain represents an outbreak (i.e., outbreak-representative strain), and a cluster is a molecular cluster of outbreaks (i.e., cluster of outbreak-representative strains).

Because the norovirus genome shows its highest variability in the capsid, comparing sequences from this region will yield the lowest number of identical strains (*31*). Therefore, regions C and D, both located in open reading frame (ORF) 2 at the capsid gene, were our regions of choice for identification of linked outbreaks. All norovirus outbreak strains reported to the FBVE network from January 1999 through November 2008 were included if a full or partial capsid sequence was involved. This yielded 1,504 outbreak-representative strain sequences reported by all above-mentioned countries. Sequence lengths varied between 90 nt and 1,640 nt. We used sequences including ORF2 nt positions 1–300 (93%) and other targets within ORF2 nt positions 300–1,620 (7%), including full capsid genes (8%).

Assignment of Genotypes

We classified genotypes on the basis of their similarity to reference strains representing known genotypes using the norovirus typing tool (www.rivm.nl/mpf/norovirus/typingtool). In this study, the ORF2 reference set was used for genotyping.

Alignments of Strains Representing Outbreaks

Nucleotide sequence alignment and similarity calculation according to the neighbor-joining method were performed for all ORF2 outbreak strain sequences within genotypes by using Bionumerics 5.1 (Applied Maths, Kortrijk, Belgium). If sequences from non-overlapping nucleotide positions were included, these sequences were separately aligned within the involved nucleotide positions.

Analysis of Clustering Strains Representing Outbreaks

Alignments were imported into the R project version 2.8.0 (http://cran.r-project.org) for analysis in 6 steps (Figure). In step 1, the APE (*32*) and the seqinR (*33*) packages in R were used to assign numbers to clusters of identical outbreak strain sequences, according to pair-wise comparison of strains within genotypes. Cluster numbers were assigned to enable rapid and computerized linking of the large molecular and epidemiologic datasets and allow systematic statistical analysis of combined data.

In step 2, the characteristics of outbreak strain sequence clusters were compared with respect to the following aspects: frequency of clusters within genotypes, sizes of clusters, the overlapping number of nucleotides, number of countries involved, period over which outbreaks were reported, transmission modes as reported in the categories foodborne, FHB, person-borne, and UN. On the basis of available information, reported sources of infection in foodborne outbreaks were allocated to the following categories: filter-feeding bivalve shellfish (including oysters and mussels); berries (including raspberries, blueberries, and strawberries); water (including water related to food preparation, irrigation, and contaminating floods, but no shellfish or berries reported); ready-to-eat (including foods like bread, sandwiches, layer cakes, food purchased at a delicatessen, salad, but no shellfish or berries reported as one of the ingredients); and other (including self-served meals, buffet or catering, with multiple food items but none of the previous food classes reported). Outbreak strain sequence clusters that included at least 1 foodborne outbreak were selected for further analysis, and designated "possible foodborne clusters."

In step 3, we used the APE package of R to stepwise extend all possible foodborne clusters to include sequences with similarities of 99.5%, 99%, 98%, 97%, 96%, and 95%. This was done to determine the cutoff level, i.e., the level of similarity needed to recognize potentially linked outbreaks and thereby to assist in the confirmation of definitely linked outbreaks.

In step 4, p values were calculated to determine association with food for a chosen cutoff level. To do so, the frequencies of the transmission mode for each strain were considered a random draw from the frequencies of this transmission mode in the background population in

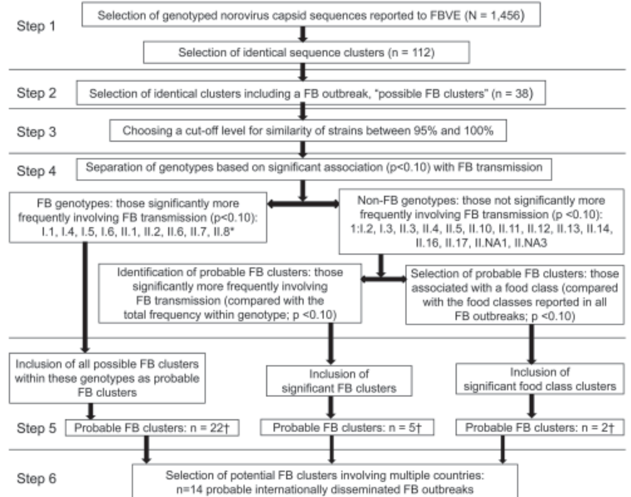

Figure. Selection of foodborne (FB) clusters of strains potentially representing internationally disseminated common-source outbreaks. Selection involved 6 steps, according to combined epidemiologic and molecular criteria. Each analyzed strain represented an outbreak. *See online Appendix Table 1, www.cdc.gov/EID/content/17/3/412-appT1.htm; †see online Appendix Table 2, www.cdc.gov/EID/content/17/3/412-appT2.htm.

the database as a null hypothesis, i.e., as random draws from a binomial distribution, and thus the probability of finding such a cluster by chance is low. For example, a cluster of 5 strains that includes 2 foodborne (i.e., 40%) has a probability of 0.02 to be found coincidentally in a total dataset containing 5% foodborne outbreaks. This cluster is then considered to be significantly associated with foodborne transmission. Such calculations were done to determine the following: 1) the association of genotypes with foodborne transmission, i.e., foodborne genotype, with the frequency of foodborne outbreaks in the genotype considered as a random draw from the total dataset; 2) association of clusters with foodborne transmission, i.e., the frequency of the foodborne mode of transmission for the specific cluster considered as a random draw from all strains in the genotype; and 3) association of clusters with a specific food class, i.e., the frequency of the food class for the specific cluster considered as a random sample from all foodborne outbreaks. These calculations were the basis for the transition from possible to probable foodborne clusters.

In step 5, the calculations of step 4 were used to narrow all possible foodborne outbreaks to probable foodborne outbreaks, i.e., those clusters that were more likely to be related to food. The clusters that were significantly and borderline significantly associated with food were selected according to 3 selection criteria: 1) all possible foodborne clusters in foodborne genotypes; and for the non-foodborne genotypes; 2) those possible foodborne clusters that were significantly or borderline significantly associated with

foodborne transmission; and 3) those possible foodborne clusters that were significantly or borderline significantly associated with a specific food class. Clusters selected through these criteria were designated probable foodborne clusters; p values were considered significant if $p<0.05$, and borderline significant if $0.05<p<0.10$. In step 6, outbreaks that could be linked and internationally disseminated were selected from probable foodborne clusters if they involved ≥2 countries.

Verification of Outcomes

The above selection criteria were applied to the FBVE database to retrospectively identify clusters of outbreaks that may have involved international dissemination of food. To verify the criteria of the approach as described above, the selected clusters were compared with the clusters previously reported to FBVE as linked outbreaks, as a measure of sensitivity of the approach (i.e., ability to detect true clusters).

Estimate of Proportion of Common-Source Foodborne Outbreaks

The frequency of linked foodborne outbreaks, both at national and international levels, was calculated as a proportion of the total number of reported outbreaks, based on the above analyses. Due to uncertainty of the causal, consequential, or coincidental relationship between outbreaks in molecular clusters, estimates were given for low, most likely, and high values of the number of linked foodborne outbreaks. Low values were calculated as the actual frequency of outbreaks in the specific clusters reported to be foodborne. High numbers were the total number of outbreaks in the specific clusters, i.e., including all reported transmission modes. For the likely values, outbreaks from unknown transmission were extrapolated proportionally to the foodborne outbreaks reported in the specific cluster. Thus, likely values were calculated as follows: for a cluster of x outbreaks containing a (foodborne), b (PTP), and c (FHB), and d (UN) outbreaks, the high value is x, the low value is a, and the likely value is

$$a+\frac{a}{a+b+c}\times d$$

Likely values with range for low and high values were subsequently compared with results of the epidemiologic overviews currently used in outbreak reporting in Europe (*24*,*25*).

Results

Assignment of Genotypes

Genotyping resulted in clustering of reported outbreak strains into 23 ORF2 genotypes for 1,456 (97%) of 1,504 sequences. For the remaining 48, sequence data provided were insufficient for assignment of a genotype.

Cluster Analysis

The degree of strain similarity and the proportion of clustering strains (Figure, steps 1 and 2) varied greatly among genotypes (online Appendix Table 1, www.cdc.gov/EID/content/17/3/412-appT1.htm). A total of 112 clusters of identical (100% similarity) outbreak strains were found, with 938 (64%) of 1,456 reported outbreaks found in clusters. Of these, 38 sequence clusters involving 654 (70%) of 938 outbreaks included at least 1 foodborne outbreak. These were designated possible foodborne clusters, i.e., possibly representing linked foodborne outbreaks (Figure, step 2).

When the cutoff for strain similarity was lowered step-wise in R (Figure, step 3), logically, the number of distinctive clusters decreased, whereas the size of each cluster increased. The similarity cutoff differed between genotypes. Six genotypes (I.1, I.4, I.5, II.1, II.5, and II.8) yielded a cluster of strains that remained distinct regardless of the cutoff used. For the other genotypes, lowering the cutoff to similarity levels of 99.5% or 99% showed a sharp drop in the number of distinct clusters, i.e., fewer clusters; as a consequence, clusters increased in size. For 7/14 genotypes the number of such clusters dropped to 50% at cutoff value of 99.5%. At 99%, this was the case for 10/14 genotypes (data not shown). Because we aimed to provide a conservative estimate for linked outbreaks for all genotypes, 100% similarity was chosen as the cutoff for further analysis steps.

Probable foodborne clusters of outbreaks were selected from 38 possible foodborne clusters based on 3 criteria for statistical association with food (Figure, step 4; online Appendix Table 2, www.cdc.gov/EID/content/17/3/412-appT2.htm): 1) twenty-two clusters in 8 genotypes (I.1, I.4, I.5, I.6, II.1, II.2, II.6, II.7, II.8) significantly or borderline significantly more often contained foodborne outbreaks, compared with the total dataset; 2) five additional clusters showed nonfoodborne genotypes for which the specific transmission mode foodborne was more frequently reported than in the genotype; and 3) two additional clusters were associated with a food class, compared with the frequency of these food classes reported for all foodborne outbreaks. Fourteen of these 29 probable foodborne clusters involved >1 country and were therefore labeled as probable internationally disseminated foodborne outbreaks (Figure, step 6).

Validation of Criteria

In the FBVE dataset of 1,456 capsid sequences, 36 outbreaks had previously been identified as linked outbreaks in 10 clusters, based on standard epidemiologic

investigation (*24*). In contrast, in the present study, 29 clusters of interest involving 122 likely linked outbreaks (range 51–166) were retrospectively identified (online Appendix Table 2). Of the 10 previously reported FBVE outbreak clusters, 8 were identified by using the approach described in this paper. These 8 clusters involved 32 likely linked outbreaks (range 18–69) and included 2 international and 3 national clusters, plus 3 clusters reported as national but containing sequences identical to those from outbreak strains reported elsewhere. The 2 FBVE clusters that were missed by our analysis involved 3 outbreaks with 3 different genotypes involved, and 3 outbreaks for which 2 different food classes were reported (ready-to-eat and other). Both food classes ended up nonsignificant for this cluster in step 5 (Figure) of the analysis.

International Clusters Potentially Linked through a Common Source

Previously, 36 of 1,456 (2.5%) outbreaks reported through the FBVE network had been linked to a common source, of which 6 (0.4%) involved events in multiple countries. Our use of the stepwise criteria described here resulted in a significant increase to 122 of 1,456 (8.4%, range low-high: 51–166) potential common-source outbreaks, of which 97 (6.7%, range 29–130) involved events in >1 country (data not shown).

Discussion

Our analysis suggests that 7% (range 2%–9%) of norovirus outbreaks reported through the FBVE network are likely to be international outbreaks with a common source. Our estimate is at least 5-fold higher than the 0.4% recognized through routine investigations. We showed that the proportion of linked foodborne outbreaks can be estimated with a sensitivity of 80% by using step-wise selection criteria combining molecular and epidemiologic information and derived from a large background dataset. The computerized linking of epidemiologic data to aligned sequences in R project for statistical computing considerably reduced the time needed for analysis and was an essential prerequisite of this novel approach. As sequencing becomes less expensive and public health databases expand, the utility of our approach for public health decision-making will increase (*34*).

Several research groups have made efforts to estimate the public health effects of norovirus and foodborne disease, finding that viral illness varies between 1/780 UK inhabitants and 1/33 US inhabitants (*1*,*35*–*37*). For Europe, we previously estimated that 21% of all norovirus outbreaks were caused by food (*38*), but that report did not consider potential (international) links between outbreaks. In our current study, we found that 2%–9% of all reported outbreaks may be linked to a common source with international distribution. Because this study was done retrospectively, we could not collect additional data to verify suspected clusters. To prove this with certainty, the analysis should be done in real time and involve more in-depth outbreak investigations to establish a risk food with epidemiologic approaches and possibly food testing. However, we do see this as a novel approach to provide the basis for estimates of the prevalence and public health consequences of foodborne disease. Past studies have used gross extrapolations of data estimating the proportion of reported noroviral disease that can be attributed to food, but have not included the effect of outbreaks. We suggest a basis for such estimates, and especially the proportion attributable to foodborne transmission.

Our approach most likely provides a conservative estimate, because it relies on identical sequence clusters and does not include outbreaks caused by strains that are phylogenetically closely related. Given the mutation rate of genotype II.4 noroviruses (*39*), closely related strains could well represent linked outbreaks. This mutation rate, as well as the similarity cutoff, may be genogroup or genotype specific (online Appendix Table 1). When a single similarity cutoff for all genotypes is used as a selection criterion, any mutation counts equally. Nevertheless, a particular mutation may indicate that strains share a common ancestor, and a mutation in a particular genotype may indicate either a longer or shorter genetic distance. Therefore, phylogenetic analysis is needed to identify additional linked outbreaks involving closely related strains.

A shortcoming of our methods is that we would miss common source events that involve >1 strain, as has been described in some examples that involved sewage-contaminated shellfish (*6*,*19*,*40*). Nevertheless, we detected 3 of 4 linked outbreaks involving multiple genotypes, which indicates that such outbreaks are likely to show other characteristics that can be captured by our criteria.

A prerequisite for our approach is the availability of combined epidemiologic and laboratory data. National surveillance systems differ in their potential for matching these data, in the intensity of surveillance, and in the attention given to foodborne outbreaks. With no special focus, foodborne outbreaks are likely to be underreported, as recognition will be complicated by rapid emergence of PTP transmission (*10*). Moreover, many persons are involved in data entry, which may have had consequences for data quality because of human error (*24*). Unevenness of data quality may hamper international comparisons to detect foodborne outbreaks (*25*). For instance, France and Denmark have been reporting primarily foodborne outbreaks, whereas other countries include a wider range of transmission modes. An added value of our approach is reflected in its finding of foodborne outbreaks in France

and Denmark and in other countries as well, e.g., the United Kingdom and Germany, which are less focused on identification of foodborne outbreaks. Despite the fact that underreporting of foodborne outbreaks in our dataset was likely, our criteria may thus provide insight into the number of foodborne outbreaks occurring in countries whose surveillance systems may miss such outbreaks.

In conclusion, combined epidemiologic and molecular analysis can recognize internationally disseminated outbreaks that may share a common foodborne source. Step-wise selection criteria can be derived from an extensive background dataset and used to retrospectively estimate the proportion of international outbreaks that share a foodborne source. Prospective use of the criteria needs to be validated through real-time data sharing and timely follow-up of outbreak clusters. Our findings nevertheless show that current surveillance has a critical gap, which can be bridged through systematic analysis of combined molecular and epidemiologic data.

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Nontuberculous Mycobacteria from Household Plumbing of Patients with Nontuberculous Mycobacteria Disease

Joseph O. Falkinham, III

To determine whether plumbing could be a source of nontuberculous mycobacteria (NTM) infection, during 2007–2009 I isolated NTM from samples from household water systems of NTM patients. Samples from 22/37 (59%) households and 109/394 (28%) total samples yielded NTM. Seventeen (46%) of the 37 households yielded ≥1 *Mycobacterium* spp. isolate of the same species as that found in the patient; in 7 of those households, the patient isolate and 1 plumbing isolate exhibited the same repetitive sequence-based PCR DNA fingerprint. Households with water heater temperatures ≤125°C (≤50°C) were significantly more likely to harbor NTM compared with households with hot water temperatures ≥130°F (≥55°C) ($p = 0.0107$). Although households with water from public or private water systems serving multiple households were more likely to have NTM (19/27, 70%) compared with households with a well providing water to only 1 household (5/12, 42%), that difference was not significant ($p = 0.1532$).

Nontuberculous mycobacteria (NTM) are opportunistic pathogens found in the environment (e.g., water and soil) and cause life-threatening infections in humans, other mammals, and birds (*1*,*2*). The incidence of NTM disease in Canada and the United States seems to be increasing (*3*–*5*). In Toronto, Ontario, Canada, NTM disease incidence rose from 1.5 to 9.0 cases per 100,000 population during 1997–2003 (*3*). The most common NTM infecting persons in the United States are *Mycobacterium avium, M. intracellulare*, and *M. avium* complex (MAC) (*6*). Infections occur in immunodeficient (e.g., HIV/AIDS) and immunosuppressed (e.g., cancer and transplant) patients and nonimmunosuppressed persons with the classic risk factors for mycobacteria infection, which include exposure to dust or smoke and underlying lung disease (*6*,*7*). Cystic fibrosis (*8*), heterozygosity for mutations in the cystic fibrosis transmembrane conductance regulator gene (*9*), and α-1-antitrypsin deficiency (*10*) predispose persons to NTM disease. Elderly, slender women lacking any of the classic risk factors for NTM disease are also at risk for NTM pulmonary disease (*11*–*13*). The major manifestation of NTM infection in the immunocompetent host is pulmonary disease, whereas disseminated disease (i.e., bacteremia) is found in patients with AIDS and other immunosuppressed persons (*6*).

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NTM, particularly *M. avium* and *M. intracellulare*, have been recovered from a variety of environmental niches with which humans come in contact, especially drinking water (*14*–*19*). NTM are not transient contaminants of drinking water distribution systems; rather, the NTM grow and persist in plumbing (*19*,*20*). For example, numbers of mycobacteria increase in pipes as the distance from the treatment plant increases (*19*). NTM cell surface hydrophobicity results in disinfectant resistance and a predilection to attach to surfaces where NTM grow and form biofilms (*21*,*22*) that further increase disinfectant resistance (*23*). Because disinfectants inhibit the competing microflora, the slow-growing NTM can grow on the available nutrients in the absence of competition. *M. avium* can grow in drinking water at concentrations of assimilable organic carbon of >50 µg/L (*24*). Thus, there is strong reason to hypothesize that NTM can colonize and persist in household plumbing.

Sources of human infection with NTM, including MAC, have been found in water (*18*) and potting soil (*25*). Notably, *M. avium* was detected in water aboard the Russian space station Mir (*26*). Recently, researchers found that the DNA fingerprints of several *M. avium* isolates

recovered from the shower of an *M. avium*–infected patient were almost identical to isolates recovered from the patient, indicating that the household water could have been the source of the patient's pulmonary disease (*27*). Despite that evidence, several publications have documented low frequency of recovery of MAC from household water samples (*17*,*28*–*30*). Such low recovery rates of *M. avium* and *M. intracellulare* could be because water samples, not biofilm, were collected. As MAC preferentially attaches to surfaces (*21*–*23*), MAC may be at low numbers in water samples. Furthermore, in the studies cited above, a low number (≤4) of samples were collected from individual households. Recovery of multiple NTM or MAC isolates is necessary because of the clonal variation of MAC (*25*,*27*). The pilot study described here isolated, enumerated, and DNA fingerprinted NTM from households of patients with NTM to test the hypothesis that household plumbing could be a source of their NTM infection.

Methods

Patients and NTM Isolates

NTM patients were recruited to participate in studies of their household water systems through the auspices of Nontuberculous Mycobacteria Research and Information, Inc. Informed consent was obtained from each participating patient, and the study was reviewed by the Virginia Tech Institutional Research Board and granted exempt status. NTM isolates from the patients, if possible, were obtained through collaborating physicians and mycobacteriology laboratories. In some instances multiple patient isolates of different species were found. A questionnaire was provided to each patient to obtain information about the household plumbing.

Household Water and Biofilm Samples

Sterile containers and swabs were sent to each collaborating patient household. Directions for collection of hot and cold water samples (500 mL) and biofilms/sediment from water taps and showerheads by using swabs were provided. If the patient thought that infection might have occurred as a result of exposure to soil, soil samples were collected. In some cases filters (fiber, activated charcoal, and reverse osmosis) were collected. All samples were returned at ambient temperatures by express courier service to the Mycobacteriology Laboratory in the Department of Biological Sciences at Virginia Tech.

Isolation and Identification of Mycobacteria

Mycobacteria in water and swab (taps and filters) samples were counted and isolated as described (*27*). Soil samples were processed as described (*25*). Most acid-fast colonies picked for identification and enumeration were small (1-mm diameter after 14 days at 37°C), unpigmented to yellow, and resembled either the transparent or opaque types previously reported (*17*). Acid-fast isolates were identified by nested PCR of the 16S rRNA gene (*31*) and PCR amplification and analysis of restriction endonuclease digestion fragments of the heat-shock protein 65 (*hsp65*) gene (*32*).

Fingerprinting Patient and Environmental Isolates

In those instances in which the *Mycobacterium* species from the patient and household water system isolates were the same, isolates were fingerprinted by repetitive sequence-based PCR (*rep*-PCR) (*33*). Matches were confirmed by use of GelCompar II software (Applied Maths, Inc., Austin, TX, USA).

Results

Household Plumbing Samples

Samples for NTM isolation were received from 31 collaborating patients throughout the United States and Canada: Arizona, California, Colorado, Connecticut, Florida, Georgia, Michigan, New Jersey, New York, Pennsylvania, Texas, Vermont, Virginia, and Wisconsin, USA; and Ontario, Canada. Six patients each had 2 residences and sent samples from each residence.

NTM Isolation

The isolates from the 31 patients with NTM infection included *M. avium* (9), *M. intracellulare* (6), MAC (11), *M. abscessus* (4), and *M. xenopi* (1). Isolates could not be obtained from 11 patients, thus preventing *rep*-PCR fingerprinting even in those instances where household isolates belonged to the same species. Thus, the total number of patient isolates available for fingerprinting was only 20. All putative *Mycobacterium* spp. isolates recovered from samples were identified, and 45% of NTM-positive households (10/22) and 1.5% of NTM-positive samples (6/394) yielded >1 NTM species (Table 1). The average number of different NTM species per household was 1.9 (range 1–5 NTM species/household). In those instances where the *Mycobacterium* species of the patient and their household plumbing isolates were the same (e.g., *M. avium*), all isolates belonging to the same species as the patient were subject to *rep*-PCR fingerprinting. Household isolates included *M. avium* (10), *M. intracellulare* (10), *M. malmoense* (5), *M. szulgai* (3), *M. chelonae* (2), *M. gordonae* (6), and 1 each of *M. scrofulaceum*, *M. terrae*, and *M. trivale* (Table 1). Samples were coded with the first 2 or 3 letters representing each patient, a letter representing sample type (W, water; Sw, swab [biofilm]; S, soil), a number for sample number from a household collection, and the final number for the isolate from the sample; thus,

Table 1. Characteristics of NTM isolated from samples from household plumbing of patients with NTM infection, 2007–2009*

Characteristic	Value
No. patients	31
No. households sampled	37†
Households with NTM	22/37 (59)
Households with >1 NTM species	10/22 (45)
Total no. samples collected	394
Samples with NTM	109/394 (28)
Samples with >1 NTM species	6/394 (1.5)
Households with NTM of same species as patient	17/37 (46)
Household and patient NTM share same fingerprint	7/17 (41)

* Values are no. positive results/no. samples in category (%) except as indicated. NTM, nontuberculous mycobacteria.
†Six patients had 2 residences and submitted samples from each.

ML-W-6-2 is the second water isolate from the sixth sample collected from patient ML's household.

NTM were isolated from water, biofilm, filter, or soil samples from 22 (59%) households sampled and from 109 (28%) of 394 samples. There was a positive correlation between the number of samples collected per household and the number of NTM-positive samples (r = 0.4581). In 8 households >50% of the samples yielded NTM, and in 7 households no NTM were isolated. Seventeen of the 37 household sample collections had at least 1 sample that yielded an NTM isolate that belonged to the same species as that of the patient. Among those 17 households, at least 1 NTM isolate from 7 households exhibited the same *rep*-PCR fingerprint as that of the patient. Specifically, the Figure illustrates matching *rep*-PCR band patterns of patient isolate ML-P-1 (lane 3) and shower water isolate ML-W-6-2 (lane 4) from the patient's home and patient isolate TC-P-1 (lane 10) and tap water isolate TC-W-2-2 (lane 12) from the patient's home. Matches were confirmed by use of GelCompar II software (Applied Maths, Inc.). Furthermore, the Figure also illustrates the relative similarity in *rep*-PCR band patterns of patients and their household isolates and the wide differences between isolates of different patients (compare lanes 3–4, lanes 7–8, and lanes 10–12). On the basis of diversity of band patterns and the number of bands (7–14 bands), the results confirm the discriminatory power of *rep*-PCR fingerprinting (*32*). The percentage of fingerprint matches may be an underestimate because patient isolates could not be obtained for 11/31 patients, all of whom had MAC infections.

The frequency of NTM recovery from water (47/195, 24%), biofilm (46/165, 28%), filters (4/12, 33%), and soil samples (3/17, 18%) did not differ markedly. The highest numbers of NTM, as CFUs, were recovered from biofilms (10,371 CFU/cm^2), with lower numbers from filters (1,987 CFU/cm^2), soils (1,500 CFU/g), and water (157 CFU/mL). Most biofilm samples were collected by swabbing either the inside of a water tap or showerhead with a sterile swab that was immediately placed in 2 mL sterile tap water. Because the samples were shipped immediately after collection, there was little opportunity for the NTM numbers to change.

Household Plumbing Characteristics as Determinants of NTM Presence

Review of the responses to the NTM patient questionnaire led to identification of 2 factors that seemed to influence NTM in household samples. Households with water heater temperatures ≤125°C (50°C) were more likely to yield NTM (17/20, 85%) compared with households in which water temperature was ≥130°F (55°C) (6/15, 40%) (Table 2). That difference was significant (p = 0.0107; relative risk 2.125, by Fisher exact test). Although households with water from a public or private

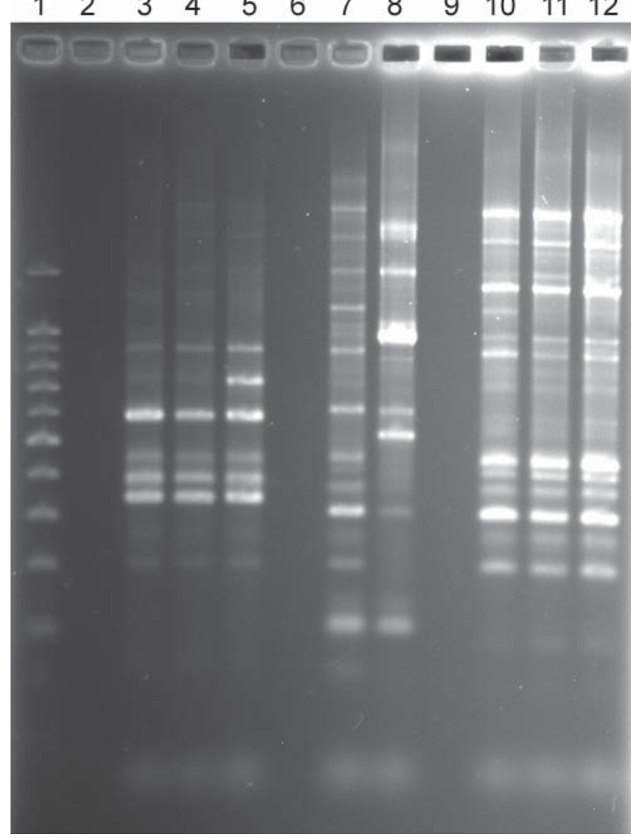

Figure. Repetitive sequence-based PCR fingerprint patterns of nontuberculous mycobacteria isolates from patients and household plumbing. Lane 1, 100-bp ladder; lane 2, no DNA control; lane 3, patient *Mycobacterium avium* isolate ML-P-1; lane 4, patient ML household *M. avium* shower water isolate ML-W-6–2; lane 5, patient ML household *M. avium* bathtub tap water isolate ML-W-8–3; lane 6, no sample; lane 7, patient *M. avium* isolate SC-P-3; lane 8, SC patient household *M. avium* water isolate SC-W-1-1; lane 9, no sample; lane 10, patient *M. avium* isolate TC-P-1; lane 11, TC household *M. avium* humidifier water isolate TC-W-4–1; lane 12, TC household *M. avium* bathroom tap water isolate, TC-W-2–2.

Table 2. Influence of water heater temperature on presence of NTM in samples from household plumbing of patients with NTM infection, 2007–2009*

Characteristic	No. households		
	NTM positive	NTM negative	Total
Water heater temperature			
≤125°F (≤50°C)	17	3	20
≥130°F (≥55°C)	6	9	15
Total no. households	23	12	35

*NTM, nontuberculous mycobacteria.

water system were more likely to have NTM (19/27, 70%) compared with households with water from a well (5/12, 42%) that difference was not significant (p = 0.1532; relative risk 1.689 by Fisher exact test) (Table 3).

Discussion

The data document the relevance of household water as a source of NTM infection. Seven (41%) of the 17 patients from whom isolates were obtained were infected with an NTM strain having the same DNA fingerprint as at least 1 NTM isolate from their household plumbing. Several characteristics of household water and plumbing are conducive to NTM survival and growth. Specifically, residual disinfectant selects for disinfectant-resistant NTM (*23*), pipe surfaces offer opportunities for biofilm formation (*21–23*), and low organic matter content permits growth of the oligotrophic NTM (*22*,*24*).

The frequency of samples yielding NTM (28%) reported is almost identical to the frequency of *Mycobacterium* spp. 16S rRNA sequences in biofilm (swab) samples collected from showers across the United States (*34*). In as much as that culture-independent study (*34*) did not collect samples specifically from households of NTM patients, apparently NTM are quite frequent in household water and plumbing across the United States and Canada and are not unique to household plumbing of NTM patients. In addition to exposure, host factors (*6–10*) are influential factors in the acquisition of NTM disease. For the study reported here, NTM patient contamination of samples was unlikely because the patients were either free of NTM in sputum or were continuing antimycobacteria therapy; none were persistently sputum positive. The low frequency of recovery of NTM by other studies (*17*,*28–30*) was likely because a low number of samples were collected from households. As shown here, only 28% of household samples yielded NTM, and there was a positive correlation between the number of samples collected and the recovery of NTM from household samples.

Table 3. Influence of water source on presence of NTM in samples from household plumbing of patients with NTM infection, 2007–2009*

Characteristic	No. households		
	NTM positive	NTM negative	Total
Water source			
Public or private piped	19	8	27
Well	5	7	12
Total no. households	24	15	39

*Two households received water from a piped system and a well. NTM, nontuberculous mycobacteria.

In addition to documenting the presence of NTM in households across the United States, the data from this pilot study with its relatively small sample size suggest that water heater temperature and water source could be factors influencing NTM presence. NTM were less frequently recovered from household samples whose water heater temperature was ≥130°C (≥55°C). The relative risk of NTM presence was 2.125 for households whose water heater temperature was ≤125°C (≤50°C). In fact, 6 of the 7 households whose patient and plumbing isolates shared identical *rep*-PCR patterns had water heater temperatures ≤125°C (≤50°C). That association correlates with the temperature sensitivity of NTM species. For example, the time required to kill 90% of *M. avium* cells is 1,000 min at 50°C but only 54 min at 55°C; similar times were measured for *M. intracellulare* (*35*). High water heater temperatures have been associated with low numbers of *Legionella* spp. in household and other building plumbing (*36–39*).

It would follow that persons infected or at risk for NTM disease, e.g., slender elderly persons or cystic fibrosis transmembrane conductance regulator gene heterozygotes (*8–13*), consider increasing water heater temperatures. Households whose water came from a public or private water system were more likely to have NTM in household water than those whose water source was a well (p = 0.1532, relative risk = 1.689). Although not significant, that result is consistent with the fact that NTM are seldom detected in groundwater (*40*). This pilot study will be followed by an investigation to assess the influence of a variety of household plumbing characteristics in households of additional NTM patients and their neighbors.

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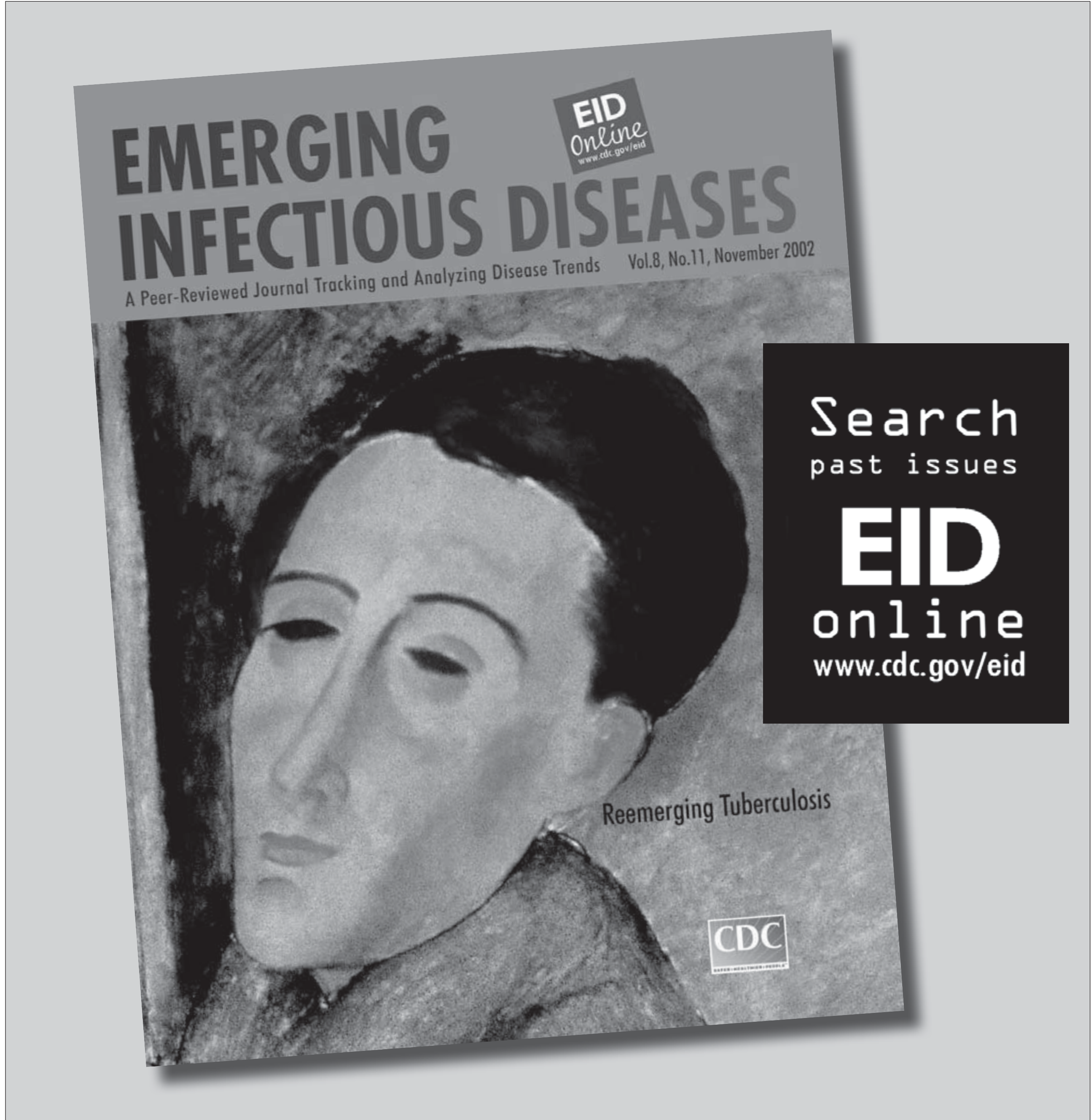

Tuberculosis Outbreak Investigations in the United States, 2002–2008

Kiren Mitruka, John E. Oeltmann, Kashef Ijaz, and Maryam B. Haddad

To understand circumstances of tuberculosis transmission that strain public health resources, we systematically reviewed Centers for Disease Control and Prevention (CDC) staff reports of US outbreaks in which CDC participated during 2002–2008 that involved ≥3 culture-confirmed tuberculosis cases linked by genotype and epidemiology. Twenty-seven outbreaks, representing 398 patients, were reviewed. Twenty-four of the 27 outbreaks involved primarily US-born patients; substance abuse was another predominant feature of outbreaks. Prolonged infectiousness because of provider- and patient-related factors was common. In 17 outbreaks, a drug house was a notable contributing factor. The most frequently documented intervention to control the outbreak was prioritizing contacts according to risk for infection and disease progression to ensure that the highest risk contacts were completely evaluated. US-born persons with reported substance abuse most strongly characterized the tuberculosis outbreaks in this review. Substance abuse remains one of the greatest challenges to controlling tuberculosis transmission in the United States.

Among the major challenges in achieving tuberculosis (TB) elimination in the United States are preventing, detecting, and responding to TB outbreaks. Identifying high-risk settings and applying effective control measures to reduce TB transmission are basic principles of TB control. Since the 1985–1992 TB resurgence in the United States, enhanced infection control measures in health care facilities have successfully reduced nosocomial transmission and outbreaks (*1*). However, outbreaks in community settings have continued to occur, calling for increased vigilance in understanding and controlling TB transmission (*1*).

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When health departments determine that they have exceeded their surge capacity to control a TB outbreak, the Centers for Disease Control and Prevention (CDC) Division of Tuberculosis Elimination may be invited to assist. During an onsite investigation lasting ≈2–3 weeks, CDC works closely with its public health partners to describe the epidemiology of the outbreak, find additional cases, identify transmission sites, prioritize contacts for screening, and implement control measures (*2*). To understand circumstances of TB transmission that tax local resources, we present an overview of US TB outbreaks during 2002–2008 for which CDC assisted in the investigation. We identified the outbreak population, outbreak contributing factors, the most common transmission sites, and interventions used to control these challenging outbreaks.

Methods

Inclusion Criteria and Data Sources

We conducted a retrospective review of TB outbreak investigations in the United States for which CDC provided onsite assistance during 2002–2008. Included in the review were outbreaks having documented evidence of *Mycobacterium tuberculosis* transmission with ≥3 culture-confirmed TB cases linked by both genotype and epidemiology. Genotyping methods included spoligotyping (all years) and either restriction fragment length polymorphism (2002) or 12-locus mycobacterial interspersed repetitive units (MIRU) (2003–2008). Linkage by epidemiology meant known exposure to another outbreak patient by sharing enclosed airspace in the same period. Linkage by genotype required matching results by whichever genotyping methods were used for that outbreak (generally by spoligotype and either restriction fragment length polymorphism or 12-locus MIRU).

We systematically abstracted data on case characteristics, outbreak contributing factors, transmission sites, and interventions (including contact investigation) from reports written by CDC teams upon return from outbreak investigations. All reports were uniformly written (with background, methods, results, discussion, and conclusion sections) and included aggregate data on demographic, clinical, and social risk factors of cases, epidemiologic linkages, genotyping results, and contact investigations. These data covered the period of the onsite investigation and were either provided by the health departments or collected by CDC as part of the investigation. The reports also discussed the cause of the outbreak and were sent back to the respective local and state health departments with recommendations to further assist in achieving control of the outbreak. We referred to any subsequent presentations and publications describing the investigation (*3*–*19*) to check data quality and accuracy of outbreak contributing factors and transmission sites. For any discrepancy in number of outbreak cases, we deferred to the CDC reports to ensure consistency in the period of data collection across all outbreaks included in the review.

Definitions

We defined outbreaks based on CDC guidelines for contact investigation as detection of TB disease among ≥2 persons exposed to a person with infectious TB (*20*), i.e., ≥3 linked cases within 2 years. The standard National TB Surveillance System variables were abstracted for all patients (*21*). Demographic, medical, and social characteristics were ascribed to an outbreak if ≥50% of the patients involved in the outbreak had that particular characteristic. For outbreak contributing factors, hotspots, and interventions, discrete categories were created based on recurring themes in the reports to enable systematic abstraction and quantification of these variables. Because multiple outbreak contributing factors and a large number of interventions were documented in CDC reports for each outbreak, we abstracted 2 factors considered by the onsite investigators to be key to fueling the outbreak and 3 interventions not already being used that were either used by the CDC team onsite or recommended to be pivotal to the overall control effort. The frequency that each category was encountered was totaled to quantify these outbreak variables. Whenever feasible, a member of the original team investigating the outbreak was consulted to review the accuracy of abstracted data.

Prolonged infectious period was defined as >3 months between symptom onset and the date that effective treatment had been administered for 2 weeks (*20*). Delayed diagnosis was defined as >2 months between symptom onset and date that the patient first sought care for TB symptoms or that TB treatment was initiated (*22*,*23*). Incomplete contact investigations meant inability to either locate or complete evaluation of contacts because of limited resources or a hard-to-reach population. Hotspots were defined as transmission sites where ≥2 outbreak patients had spent substantial time together, as determined by local public health investigators. Drug house was defined as a venue characterized by the sale or use of illicit drugs. Household was defined as a residential location without documented illicit drug use.

Results

Of the 51 TB investigations in which CDC participated during 2002–2008, a total of 27 met the inclusion criteria. Twenty-four were excluded for the following reasons: 12 investigations (2 included patients with organ transplants) involved <3 cases; 5 had <3 cases linked by genotype and epidemiology; 5 were international investigations; and 2 had insufficient data in CDC reports.

Patient Summaries

The 27 outbreaks included in the review involved 398 patients (median 10 patients/outbreak, range 3–35 patients). Of these patients, 364 (91%) were US-born and 50 (13%) were <15 years of age (Table 1). Three hundred thirty-three (84%) had pulmonary disease, including 204 (61%) and 284 (85%) with smear-positive and culture-positive TB, respectively. Eighty-nine percent of isolates (253 of 284) were susceptible to first-line TB medications (Table 1). Of the 197 patients for whom the reason for initial TB evaluation was documented, 74 (38%) were evaluated because of TB symptoms, and 57 (30%) were detected as part of a contact investigation. Ninety-nine (25%) patients required hospitalization, and 23 (6%) died. Infectious periods were documented for 172 patients; the mean and median infectious periods were 6 and 5 months, respectively (range 1–56 months).

Most patients did not have established medical risk factors for TB (Table 2). Although the total number of patients tested for HIV was not available, HIV infection was documented for 45 patients (12%). Multiple social risk factors for TB were documented: 233 (58%) patients reported alcohol abuse or use of illicit drugs, 126 (32%) had a history of incarceration, and 78 (20%) had a history of homelessness. Sixteen (4%) patients were documented to have a previous diagnosis of TB; of these, 7 (44%) had received incomplete TB treatment.

Spoligotype and MIRU genotype data were available for 22 of 27 outbreaks. Three outbreaks involved a Beijing strain (spoligotype 000000000003771, MIRU 223325173533); the remaining 19 had genotypes that differed from each other. The most frequent strain lineage in these outbreaks was EuroAmerican.

Table 1. Characteristics of patients in CDC–investigated TB outbreaks, United States, 2002–2008

Characteristic	No. (%) patients
Total	398 (100)
Demographics	
US-born	364 (91)
Black	265 (67)
Male sex	259 (65)
White	66 (17)
Age <15 y	50 (13)
Hispanic	31 (8)
Clinical signs and outcomes	
Pulmonary TB	333 (84)
Cavity on chest radiograph	122 (37)
Sputum acid-fast bacilli smear positive	204 (61)
Sputum culture positive	284 (85)†
Susceptible to first-line tuberculosis medications	253 (89)
Hospitalization	99 (25)
Death	23 (6)

*TB, tuberculosis; CDC, Centers for Disease Control and Prevention.
†An additional 10 patients had non-sputum specimens that were culture-positive.

Outbreak Summaries

US birth and substance abuse were predominant features of outbreaks (Table 3). On the basis of ≥50% of outbreak patients having a particular characteristic, the criterion used to ascribe a characteristic to an outbreak, 24 of the total 27 outbreaks were characterized by US-born persons, and 18 outbreaks by patients with reported substance abuse. Fourteen (52%) outbreaks were characterized by US-born men who used alcohol to excess or illicit drugs, i.e., marijuana, cocaine, methamphetamine, or heroin. All 8 outbreaks characterized by patients with incarceration histories and the 4 characterized by homelessness had a predominance of patients with reported substance abuse.

Of the 24 outbreaks that occurred among predominantly US-born persons, 21 outbreaks involved substance abuse. For 17 (71%) outbreaks, ≥50% of patients reported substance abuse; for 2 additional outbreaks, ≥40% of patients reported substance abuse; and in 2 others, the source patients who reported substance abuse had prolonged infectious periods during which TB was transmitted. In all, 21 (87%) of 24 outbreaks of predominantly US-born patients could thus be characterized as being related to substance abuse. The remaining 3 outbreaks associated with US-born persons were characterized by delayed diagnosis that resulted in transmission in a health care facility (*6*), among family members of a recently incarcerated patient (*7*), and among family members of an undocumented worker (*17*).

Three of the 27 outbreaks occurred among predominantly foreign-born persons. In 1 of these outbreaks, all patients engaged in substance abuse, and although these patients were foreign-born, they had been in the United States for more than a decade (*18*). The other 2 outbreaks among foreign-born persons involved transmission in school, church, and household settings. In each outbreak, the foreign-born patients did not access health care (caused by, in 1 outbreak, fear of repercussions for being undocumented, resulting in multidrug-resistant TB transmission among family members) (*17*).

Outbreak Contributing Factors and Hotspots

Table 4 describes the most common outbreak contributing factors, which for 24 outbreaks was prolonged infectiousness. In 4 outbreaks in which patients delayed seeking medical attention for their TB symptoms, in 7 where provider-related diagnostic delays occurred, and in 1 where both types of delay occurred, ≥40% of patients had reported substance abuse. Incomplete contact investigations because of limited resources or a hard-to-reach population contributed to 10 outbreaks.

Drug house was the most commonly identified hotspot (17 outbreaks), followed by homeless shelter (n = 5), correctional facility (n = 4), household (n = 4), workplace (n = 4), church (n = 3), bar (n = 2), school (n = 1), and automobile (n = 1). Ten of the 17 drug houses were primarily residences with extended family members, and the other 7 were largely venues where unrelated persons gathered strictly for the use of illicit substances. In the 4 household outbreaks, crowded living conditions among extended families (primarily foreign-born in 2 outbreaks) were the main TB risk factors.

Interventions

The most frequently documented intervention to control the outbreak was to prioritize contacts based on

Table 2. Tuberculosis risk factors for patients in CDC–investigated TB outbreaks, United States, 2002–2008*

Risk factor†	No. (%) patients
Total	398 (100)
Medical	
HIV co-infection	46 (12)‡
Diabetes	23 (6)
Immunosuppression (not HIV associated)	14 (4)
History of TB	16 (4)
Incomplete treatment	7 (44)
Social	
Any substance abuse	233 (58)
Alcohol abuse	204 (51)
Nonintravenous drug use	117 (29)
Intravenous drug use	19 (5)
Incarceration history§	126 (32)
Homelessness	78 (20)

*TB, tuberculosis; CDC, Centers for Disease Control and Prevention.
†As documented in CDC reports of onsite investigation with information generally gathered through patient chart reviews or interviews.
‡Minimum estimate because complete data on the number of patients tested were not available.
§Time frame before TB diagnosis not always documented in CDC reports. The National Tuberculosis Surveillance System collects data on incarceration at time of TB diagnosis.

Table 3. Predominant characteristics of CDC–investigated TB outbreaks, United States, 2002–2008*

Characteristic	No. (%) outbreaks†
Total	27 (100)
US born	24 (89)
Male sex	22 (81)
Substance abuse (alcohol/drugs)	18 (67)
Acid-fast bacilli smear positive	17 (63)
Non-Hispanic black	16 (59)
Incarceration history	8 (30)
Cavitary disease on chest radiograph	7 (26)
Non-Hispanic white	4 (15)
Homelessness	4 (15)
Hispanic	3 (11)
HIV co-infection	1 (4)

*TB, tuberculosis; CDC, Centers for Disease Control and Prevention.
†Outbreak had ≥50% of patients with the select characteristic.

risk for infection and progression to disease to ensure that the highest risk contacts were completely evaluated (14 outbreaks). This intervention was necessitated by the large number of contacts identified; contact investigation of 398 patients had generated 16,559 contacts. Of these contacts, 10,142 (61%) had been evaluated by the time of the onsite investigation; 2,128 (21%) were found to have latent TB infection (range 4%–65% per outbreak). Other frequently used interventions included educating community health care providers, e.g., emergency departments, to be vigilant for TB in patients seeking treatment at their facilities (13 outbreaks), and location-based screening, which involves offering TB screening to potential contacts at that particular outbreak's hotspot or other convenient location (10 outbreaks).

Discussion

US-born persons who reported substance abuse most strongly characterized the TB outbreaks in this review: 24 of the 27 total outbreaks involved primarily US-born patients, and 19 of these outbreaks involved ≥40% of patients with reported drug or alcohol abuse. This predominance of substance abuse suggests that it remains one of the greatest challenges to controlling TB transmission in the United States.

Table 4. Factors contributing to 27 CDC–investigated TB outbreaks, United States, 2002–2008*

Factor	No. outbreaks†
Prolonged infectious period	24
Provider related	
Delayed diagnosis	12
Inappropriate treatment	2
Patient related	
Delayed diagnosis because of late access to care	6
Nonadherence with treatment	5
Mistrust or fear of public health system	6
Incomplete contact investigation	10
Crowded setting with high-risk population	7

*TB, tuberculosis; CDC, Centers for Disease Control and Prevention.
†Categories not mutually exclusive.

Because this descriptive review of TB outbreaks in the United States was restricted to investigations that prompted public health jurisdictions to request CDC assistance, it might lack generalizability to all TB outbreaks in the United States. Outbreaks involving hard-to-reach populations, such as those involving substances abuse or homelessness, with a tendency to overwhelm local public health resources, might be overrepresented. On the other hand, social risk factors such as substance abuse that are based on self-reported behavior might have been underdisclosed because of associated social stigma. Data on key medical risk factors such as HIV and diabetes might have been pending or missing during an investigation and therefore not systematically included. Because genotyping might not have been conducted on every culture-positive case in the jurisdictions affected by these outbreaks, especially during the first 2 years of this review, some cases could have been missed, underestimating the scope of these outbreaks. Despite these limitations, characteristics found to be associated with intense TB transmission are consistent with findings in the previous literature.

Although the case rate is 10× higher among foreign-born than among US-born persons, (*21*), this disparity was markedly lacking in our review; 91% of outbreak patients were US born. Prior studies have demonstrated that recent transmission occurs mainly among US-born persons, with foreign-born persons more likely to develop reactivation of latent TB infection acquired before immigration (*24–26*). Similar to other studies (*25–27*), our few examples of TB outbreaks among immigrants were all associated with crowded living conditions and lack of access to medical care, whereas outbreaks that involved mainly US-born persons were associated with substance abuse and other risk factors, such as homelessness and incarceration.

Among nationally reported TB cases, substance abuse has been estimated to be the most prevalent modifiable TB risk factor, reported by 29% of US-born vs. 8.3% of foreign-born patients (*28*). In our overview, 58% of outbreak patients self-reported substance abuse. Consistent with national TB surveillance regarding substance abuse, alcohol was the most commonly reported substance. Alcohol has been documented to increase the risk for TB exposure, susceptibility to infection, and progression to active disease (*29*,*30*). Contact investigations among bar patrons have yielded latent TB infection rates of 40%–50% (*29*,*31*), highlighting the transmission risks in this population. Failure by contacts who abuse alcohol to be treated for latent TB infection can prolong outbreaks if active TB subsequently develops in these persons and they then serve as additional sources of transmission (*4*).

Substance abuse is a long-established risk factor for TB infection and disease (*32*,*33*), but in recent years its role in fueling TB transmission has also been recognized (*10–12*,*14*,*15*,*18*,*19*,*28*,*29*,*31*,*32*). Persons who report substance abuse are associated with increased TB transmission because of sociobehavioral and clinical TB risk factors. First, persons who report substance abuse are more likely to have smear-positive disease and experience treatment failure (*28*,*34*), e.g, because of nonadherence, both of which can increase infectiousness (*20)*. The higher prevalence of smear positivity might be attributed to delayed diagnosis, or, in cases of crack cocaine use, pulmonary damage that leads to alveolar macrophage impairment and cytokine dysfunction (*34*). Second, persons who report substance abuse are likely to experience a prolonged infectious period because of delays in seeking medical care and, once they are medically evaluated, receiving a TB diagnosis (*18*,*31*,*32*). Third, sharing of drugs or alcohol often occurs in confined and poorly ventilated settings such as drug houses (*4*,*10–12*,*15*,*18*,*19*), bars (*15*,*29*,*31*), homes (*7*,*10*), and vehicles (*18*)—all of which facilitate close and prolonged contact. The most common hotspots in this review were settings in which drug use occurred; poverty, unstable housing, and overcrowded conditions exacerbated TB transmission (*4*,*7–9*,*11*,*15*,*18*,*19*). Fourth, contacts of TB patients are often difficult to identify because patients want to protect the names of contacts with whom they engage in illicit or other activities perceived to have social stigma (*10*,*28*,*31*,*32*). Our finding of an overall 21% latent TB infection rate among contacts, lower than the expected 30% (*20*), might reflect evaluation of relatively lower risk contacts whose names were easier to elicit. Finally, contacts with substance abuse can be difficult to locate, be less likely to accept and adhere to treatment, and have a greater risk for adverse reactions from medication, e.g., related to interaction of alcohol with isoniazid (*28*,*29*,*32*).

Given the predominance of patients with substance abuse in our review, it is not surprising that prolonged infectious period was the most common outbreak contributing factor. Delayed diagnosis was the most common cause (14/27 outbreaks) and has been cited as a major contributor to TB outbreaks (*3*,*7*,*11–15*, *22*,*23*,*25*,*27*,*35*,*36*). In 1 outbreak, during a 1-year infectious period, the source patient lived in 4 locations, all crowded settings, and shared illicit drugs with household members, facilitating TB transmission to 3 adults and 3 children (*11*). In another outbreak, during the 9 months that the source patient's diagnosis was delayed, the patient was in and out of jail and had multiple moves to new residences, resulting in 37 additional cases (including 10 children) across 3 counties (*9*). These examples of intense transmission occurring before a correct diagnosis was made highlight the need for educating health care providers to suspect TB when encountering either persons born abroad or domestically with social risk factors for TB, such as substance abuse, homelessness, and incarceration history (*6*,*7*,*9*,*11*,*12*,*15*,*35*). Failure to do so can lead to outbreaks that overwhelm public health resources. Additionally, raising general awareness about TB so that patients seek early medical care and know the value of completing treatment are critical to ending transmission (*22*,*36*).

This review found that incomplete contact investigation was the second most common contributing factor to TB outbreaks. When contact investigations are incomplete, a pool of latent TB infection remains, threatening to generate additional cases and cause ongoing transmission (*10*,*15*,*24*). Compounding these risks, persons who report substance abuse are more likely to be poor, homeless, and have an incarceration history—all documented TB risk factors (*5*,*11*,*13–15*,*28*,*37*,*38*). When contact investigations involve a hard-to-reach population, conventional methods of contact tracing may need to be expanded to include other approaches (*2*,*5*,*10*,*13–15*,*27*,*39*). To optimize the yield of contact investigation, the 2 interventions most frequently used in these outbreaks were prioritizing screening of contacts on the basis of TB risk (*3*,*5*,*6*,*8–11*,*18*) and offering location-based TB screening at specific venues associated with each outbreak, including bars, shelters, and drug houses (*5*,*6*,*10*,*13*,*14*). Although this intervention is resource-intensive, its benefits have been recognized in several investigations involving hard-to-reach populations (*10*,*14*,*31*). In 1 outbreak, unnamed contacts encountered at a drug house frequented by numerous TB patients were offered screening and were found to be 8× more likely to have a positive tuberculin skin test result than were named contacts (*10*).

Although this review was limited to outbreaks in which CDC was invited to assist and might not represent all TB outbreaks in the United States, it provides an opportunity to identify common themes among outbreaks which, when present, tend to challenge local public health capacity. These outbreaks featured US birth and substance abuse—factors shown to be independently associated with genotype clustering, a marker for recent TB transmission (*39*,*40*). Although TB incidence has been decreasing in the United States, its elimination will not be achieved without more effective strategies to prevent, detect, and treat TB among persons who are known to abuse substances.

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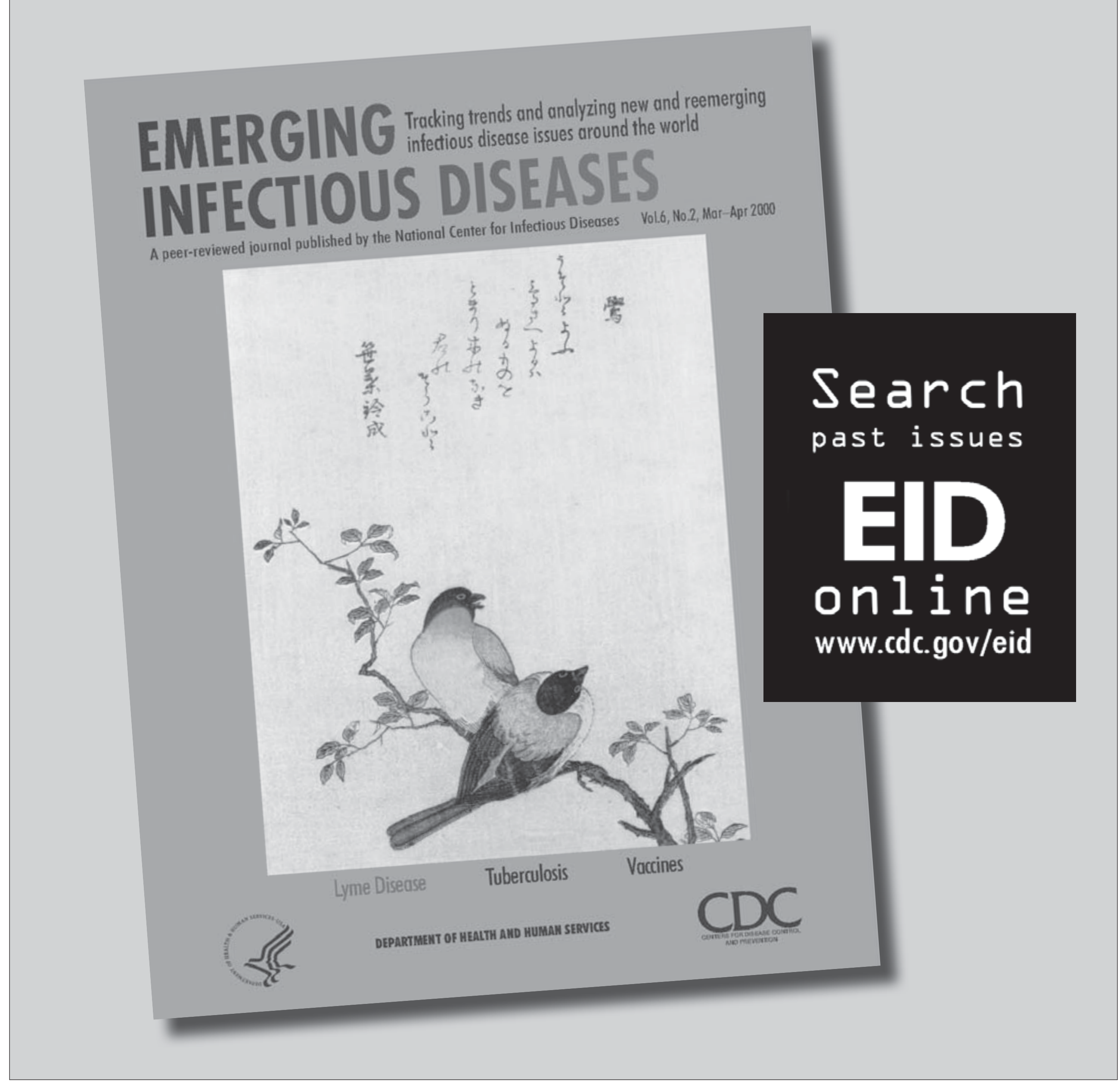

Targeted Drug-Resistance Testing Strategy for Multidrug-Resistant Tuberculosis Detection, Lima, Peru, 2005–2008

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The Peruvian National Tuberculosis Control Program issued guidelines in 2006 specifying criteria for culture and drug-susceptibility testing (DST), including district-level rapid DST. All patients referred for culture and DST in 2 districts of Lima, Peru, during January 2005–November 2008 were monitored prospectively. Of 1,846 patients, 1,241 (67.2%) had complete DST results for isoniazid and rifampin; 419 (33.8%) patients had multidrug-resistant (MDR) TB at the time of referral. Among patients with new smear-positive TB, household contact and suspected category I failure were associated with MDR TB, compared with concurrent regional surveillance data. Among previously treated patients with smear-positive TB, adult household contact, suspected category II failure, early relapse after category I, and multiple previous TB treatments were associated with MDR TB, compared with concurrent regional surveillance data. The proportion of MDR TB detected by using guidelines was higher than that detected by a concurrent national drug-resistance survey, indicating that the strategy effectively identified patients for DST.

Multidrug-resistant tuberculosis (MDR TB) is defined as infection with *Mycobacterium tuberculosis* with in vitro resistance to at least isoniazid and rifampin. The incidence of MDR TB disease was estimated to be 0.5 million in 2007, with a prevalence of as many as 2 million cases worldwide (*1*). Although no single best approach to MDR TB treatment has been recognized, rapid drug-susceptibility testing (DST) and prompt initiation of effective treatment are achievable goals. Ideally, treatment is based on timely, accurate DST, but if universal DST is not possible or not yet available, the national TB control program can prioritize patients at increased risk for MDR TB.

Rapid DST methods should minimize delays to initiation of appropriate treatment (*2*,*3*). Numerous assays have been developed that have characteristics suitable for use in low-income settings, including low cost, modest technical demand, and high accuracy (*4*–*6*). The nitrate reductase assay (NRA), also known as the Griess method, has demonstrated acceptable sensitivity, specificity, and speed compared with conventional DST and rapid phenotypic DST methods (*7*,*8*). This phenotypic assay was developed in Russia as a low-cost drug-susceptibility test that can be used in areas of moderate technical capacity (*9*). The method is based on a nitrate-reductase colorimetric reaction that uses Lowenstein-Jensen (LJ) medium prepared with antimicrobial drugs (*9*). Although initially validated as an indirect method, it was implemented as a direct method by the Peruvian National Institute of Health (INS) (*10*). The NRA yields drug-susceptibility information to isoniazid and rifampin 21–28 days after inoculating a smear-positive sputum sample (direct method) or 8–10 days after obtaining a positive culture (indirect method) (*10*). The pooled sensitivity and specificity of the NRA (on culture isolates and sputum) have been reported to be 97% and 100% for rifampin and 96% and 99% for isoniazid (*11*). The pooled sensitivity and specificity of direct NRA have been reported to be 99% and 100% for rifampin and

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94% and 100% for isoniazid (*12*). A recent comparison of 4 rapid DST methods with conventional DST in the context of a clinical trial suggested they may be cost-effective when compared with other health interventions (*13*). On the basis in part of these data, the World Health Organization (WHO) recently endorsed the use of NRA for screening patients at risk of MDR TB (*14*).

Despite the development of promising commercial and noncommercial rapid methods for MDR TB diagnosis, how to implement those methods under program conditions remains largely unaddressed. DST performance in validation studies differs greatly from performance integrated within a TB control program. Furthermore, the performance of any method under program conditions depends not only on assay characteristics, but also on the assets of the laboratory network and National Tuberculosis Control Program (NTP) guidelines which define criteria for performing DST.

To address this gap, we evaluated the effects of a programmatic strategy for rapid screening for MDR TB among risk groups specified by the Peruvian NTP in April 2006 (*15*). At that same time, decentralized, district-level MDR TB screening was pilot tested in 2 district laboratories in Lima, Peru. In collaboration with the Peruvian NTP and the Peruvian National Reference Laboratory (NRL), we evaluated the effectiveness of these combined strategies for detecting MDR TB. We report the proportion of drug resistance among risk groups based on screening high-risk patients as defined by explicit criteria, including rapid methods of DST in one of the first countries to implement this strategy.

Materials and Methods

Study Setting and Program Description

The prevalence of TB in Peru was 38,000 cases, and the incidence of TB in Peru was 126 cases/100,000 population per year, by 2007 estimates (*16*). The most recent national surveillance data indicate 5.3% of new TB cases and 24% of previously treated TB cases are MDR TB (*16*). In 1996, a collaborative effort to provide individualized treatment by using second-line drugs for MDR TB in northern Lima was established by Partners In Health, Socios En Salud, Harvard University, the Massachusetts State Laboratory Institute, the Peruvian NTP, and the Peruvian INS (*4*). In October 1997, the Peruvian NTP began a standardized treatment regimen including directly observed therapy with second-line drugs for patients in whom first-line drugs failed (*17*). Only 48% of these patients were treated successfully. During 2005 and 2006, the Peruvian INS and NTP transferred the capacity for DST to first-line TB drugs from the central level at the NRL to 2 district-level reference laboratories in Lima as a prelude to decentralizing these services to all major provinces (*4*). Simultaneously, the NTP issued national guidelines codifying criteria for MDR TB screening on the basis of known and suspected risk factors for MDR TB. In addition, these guidelines recommended the use of a more aggressive empiric MDR TB treatment regimen, including 5 second-line drugs for those persons with suspected MDR TB pending DST results. These programmatic and laboratory efforts resulted in an integrated strategy to diagnose and treat MDR TB cases in a timely and aggressive manner. During this period, we evaluated the effectiveness of selection criteria for DST in the first 2 laboratories (for health districts Lima Ciudad and Lima Este) where the NRA was implemented.

For persons in whom DST to first-line drugs confirmed drug resistance to isoniazid or rifampin, or both, the same isolate would be sent to the NRL for testing to a full panel of 5 first-line drugs and 5 second-line drugs. DST results were conveyed to health center providers by paper or electronic communication, and patients were evaluated with DST results to determine whether further regimen modification was needed (*18*). Details of treatment regimens have been described elsewhere (*19*). All patients diagnosed with TB were provided directly observed therapy free of charge through the NTP.

Study Patients and Enrollment Period

The patients enrolled in this cohort had suspected TB with respiratory symptoms living in 2 districts of Lima, Peru, Lima Ciudad or Lima Este, who met Peruvian NTP guidelines for DST referral as elaborated in Table 1. There were no exclusion criteria.

Enrollment Methods

Patients were identified by health care workers at their local health care establishments, and their sputum samples were sent to the reference laboratory for DST. Because all sputum samples for DST were sent to the district laboratories, subjects eligible for enrollment were identified by this referral. Study personnel visited each district laboratory on a regular basis to review sample referrals and confirm that all eligible subjects had been identified. Large, busy health centers were visited weekly and smaller, rural health centers were visited at least monthly for review of patient medical records. This method was used to confirm that all patients were included who were eligible without duplications. In Lima Ciudad, patients were enrolled from January 2005 through March 2008. In Lima Este, patients were enrolled from May 2005 through May 2008.

Drug Susceptibility Methods

The scale-up of MDR TB laboratory services in Peru, including expansion of the BACTEC-460 system (Becton, Dickinson and Company, Sparks, MD, USA) and NRA

(Griess) method for rapid first-line DST are described elsewhere (*4*,*5*,*20*). The Peruvian NRL performed BACTEC-460 culture and DST on paucibacillary and smear-negative samples, prioritizing pediatric cases, HIV-positive persons, and health care workers. The scale-up of second-line conventional DST at the NRL by the indirect agar plate proportion method has also been described elsewhere (*4*). The district reference laboratories cultured sputum specimens processed with 4% NaOH on Ogawa medium without centrifugation. During 2005, BioSafety Level 3 working conditions were established in 2 district reference laboratories, and these laboratories implemented DST on LJ medium for first-line drugs by using the indirect proportion method. Each procedure was validated by each laboratory through comparison with the Peruvian NRL and the Massachusetts State Laboratory Institute. Subsequently, the NRA was implemented in Lima Ciudad in December 2005 and in Lima Este in March 2007. The study spanned pre- and postintervention periods: January 2005 through November 2008 for Lima Ciudad and May 2005 through November 2008 for Lima Este. The NRA was used for rapid screening of smear-positive specimens from patients with the risk factors outlined in Table 1. Sputum specimens were processed with 2% NaOH/N-acetyl-L-cysteine, centrifuged at 3,000 × *g*, cultured on LJ medium, and simultaneously inoculated on modified LJ medium for the NRA to detect isoniazid and rifampin resistance.

Data Collection

A team of trained data recorders prospectively collected data using standardized forms. Sources of data included patient charts and laboratory registries and databases. HIV status was routinely recorded for TB patients. Available chest radiographs were reviewed by TB physicians who used standardized criteria to identify the type and location of radiographic abnormalities. Chest radiograph data were included if the radiograph was performed ≤1 year before the enrollment date or ≤1 month after the enrollment date. At baseline, clinical and sociodemographic data were recorded in addition to the risk factors outlined in Table 1. Data were entered into an Epi Info version 3.4.3 database (Centers for Disease Control and Prevention [CDC], Atlanta, GA, USA).

In addition to the data prospectively collected in this study, we used national and regional data from Peruvian NTP surveillance on drug resistance that were collected as part of the WHO Fourth Global Report on Anti-Tuberculosis Drug Resistance in the World (*21*). Surveillance and laboratory methods are described in more detail in the WHO report (*21*). For national data, we used all data in the NTP surveillance; for regional data, we included samples collected from health establishments served by the Lima Ciudad and Lima Este laboratories, which corresponded to the same catchment area as our study (Ministerio de Salud, unpub. data).

Patients were identified as having MDR TB if they had a positive culture for *M. tuberculosis* and DST results showed resistance to at least isoniazid and rifampin. Extensively drug-resistant TB was defined as resistance to at least isoniazid, rifampin, any fluoroquinolone, and ≥1 of 3 injectable second-line drugs (amikacin, capreomycin, or kanamycin). Monoresistance was defined as drug-resistance to isoniazid or rifampin, but not both drugs. Patients were considered to have drug-susceptible TB if their isolate was susceptible to both isoniazid and rifampin. Baseline refers to DST data at the time of referral, i.e., testing performed on sputum samples collected within 30 days of study enrollment. If baseline drug resistance data were not available for both isoniazid and rifampin (e.g., because of a culture-negative sample or because of contamination), the patient was considered to have no DST result.

Statistical Analysis

All analyses were performed by using STATA/IC version 10.1 (StataCorp LP, College Station, TX, USA). The χ^2 test or Fisher exact test was used to calculate p values, when appropriate. Point and interval estimation for the odds ratio were performed by using the Woolf procedure or the exact method, when appropriate. The Breslow-Day test for homogeneity was used to explore for effect modification. All statistical tests were 2-sided, and significance was set at $\alpha = 0.05$.

Ethical Approval

The prospective observational cohort study providing data for this analysis was approved by institutional review boards at Brigham and Women's Hospital and the Peruvian INS. An institutional review board amendment describing the aims of this analysis was approved by the Peruvian INS on October 15, 2008, and by the Partners Human Research Committee for Brigham and Women's Hospital on September 23, 2008. This activity was approved by CDC as program evaluation and not as human subject research.

Results

A total of 1,846 patients were enrolled during the study period. Among these, 605 (32.8%) did not have baseline DST results, either due to a nonviable sample (99.2%) or incomplete resistance data for both isoniazid and rifampin (0.8%). The remaining 1,241 (67.2%) patients constitute the cohort for analysis presented here.

Of these 1,241, 419 (33.8%) had baseline MDR TB, among whom 195 (46.5%) had never been treated for TB and 224 (53.5%) had a history of previous TB treatment. Eight patients had extensively drug-resistant TB; 1 was a medical student, 1 had received prior self-administered

Table 1. Criteria for drug-susceptibility testing referral per Peruvian National Tuberculosis Control Program guidelines*

A. Newly diagnosed smear- or culture-positive patients at risk for MDR TB. Persons were eligible for enrollment if they were 1) diagnosed with smear-positive pulmonary TB, 2) had no history of TB, and 3) had ≥1 of the following risk factors:
1. Household contact of patient with documented MDR TB
2. Household contact of patient in treatment with second-line drugs
3. Household contact of patient who showed failure of TB therapy
4. Household contact of patient who died of TB within the past 2 years
5. HIV-positive by ELISA and Western blot confirmation
6. Diabetes mellitus
7. Health care worker, regardless of health care field, in the past 2 years
8. Student of health sciences in the past 2 years
9. Employee of the penitentiary system
10. Chronic treatment with corticosteroids
11. Other condition of immunosuppression
12. Adverse reaction to TB medications requiring a change in regimen
13. Hospitalization for any indication in the past 2 years lasting >15 days
B. Patients in whom first-line or second-line therapy may be failing. Persons were eligible for enrollment if they were 1) currently receiving first-line or second-line treatment, and 2) had a sputum sample collected after >2 months of treatment that was smear positive (i.e., monthly sputum collected between months 2 and 6)
C. Patients who had received ≥1 previous treatment and who did not have documented MDR TB. This included persons who:
1. Abandoned any previous regimen and now presented for retreatment
2. Relapsed after completion of any previous regimen within 6 months
3. Unsuccessful treatment with any previous regimen
4. Received multiple courses of TB treatment
5. Had a history of private or auto-administered treatment
D. Newly diagnosed smear-negative patients at risk for smear-negative MDR TB. Persons were eligible for enrollment if they were 1) suspected to have active pulmonary TB, 2) were smear negative, 3) had no history of TB therapy, and 4) had ≥1 of the following risk factors:
1. Pediatric household contact of patient with documented MDR TB
2. Pediatric household contact of patient who died of tuberculosis within the past 2 years
3. HIV positive by ELISA and Western blot confirmation

*MDR, multidrug resistant; TB, tuberculosis.

treatment, 2 had household contacts (1 was a pediatric patient and the other was an adult), and 4 were identified as suspected category I failures, i.e., failure of first-line treatment for new patients. Of these 8 case-patients, only 1 (who had received self-administered treatment) had completed previous treatment.

Descriptive characteristics of the cohort are shown in Table 2. Compared with patients with drug-susceptible TB, those with MDR TB were younger, more likely to be single, more educated, and less likely to have ever smoked. Clinically, they were less likely to have been tested by using the BACTEC-460 system and more likely to have hemoptysis. MDR TB patients and patients with drug-susceptible TB did not significantly differ with respect to their year of enrollment, gender, and history of TB treatment. Compared with patients with drug-susceptible TB, patients with monoresistant TB were younger and more likely to be single.

The most frequent risk factors prompting referral for DST among patients with new smear-positive TB were being adults with a household contact with known or suspected MDR TB (32.1%), diabetes mellitus (20.0%), and suspected category I failures (19.5%). Among previously treated patients with smear-positive TB, those with multiple (≥2) treatments (43.2%), adult household contact (18.6%), default of category I treatment (16.8%), and previously self-administered treatment (14.9%) were most frequently referred for DST. Among all patients with smear-positive TB, a single risk factor was identified in 485 (43.54%) patients, whereas 382 (34.29%), 205 (18.40%), 38 (3.41%), and 4 (0.36%) had 2, 3, 4, and 5 risk factors, respectively (data not shown).

The prevalence of MDR TB in Peru in 2007 among all TB patients, previously treated TB patients, and new TB patients is shown in Table 3 (*21*). In this national surveillance report, 8.3% of all TB patients, 5.2% of new TB patients, and 24.2% of previously treated TB patients in Peru were estimated to have MDR TB (*21*). Limiting surveillance data to the 2 districts where our cohort was enrolled, 12.4% of all TB patients, 9.9% of new TB patients, and 24.0% of previously treated TB patients had MDR TB (Ministerio de Salud, unpub. data). In our cohort of 1,241 subjects, 33.8% of all patients, 31.6% of new TB patients, and 35.8% of previously treated TB patients had MDR TB. Because national surveillance was conducted on smear-positive samples only, we compared the proportion of MDR TB among patients with smear-positive results in our cohort to prevalence of MDR TB from regional surveillance estimates. As shown in Tables 4 and 5, our cohort showed higher risk for MDR TB among new TB patients and previously treated TB patients.

When stratifying our cohort by risk group, we found that diabetes mellitus (16.8%), adult (33.5%) or child (53.9%) patients with household contacts with known or suspected MDR TB, and suspected category I failure, i.e., positive smear or culture during the second or third month of category I therapy (66.7%), were associated with significantly higher relative risks of MDR TB among patients with new smear-positive TB, when compared to regional surveillance prevalence estimates. Among the 18 patients with diabetes and new smear-positive MDR TB, 10 (55.6%) had 2 risk factors for MDR TB at the time of enrollment. Of these, 5 (27.8%) had suspected category I

Table 2. Demographic and clinical characteristics of patients with tuberculosis, by drug-resistance status, Lima, Peru, 2005–2008*

Variable	Susceptible to INH and RIF, n = 661	Monoresistant to INH or RIF, n = 161	MDR TB, n = 419	Total, N = 1,241
DST method				
Griess	318 (48.1)	87 (54.0)	208 (49.6)	613 (49.4)
Conventional	270 (40.9)	56 (34.8)	185 (44.2)	511 (41.2)
BACTEC	63 (9.5)	17 (10.6)	**23 (5.5)†**	103 (8.3)
Griess/BACTEC	10 (1.5)	1 (0.6)	3 (0.7)	14 (1.1)
Year of enrollment				
2005	175 (26.5)	44 (27.3)	130 (31.0)	349 (28.1)
2006	233 (35.3)	47 (29.2)	127 (30.3)	407 (32.8)
2007	178 (26.9)	48 (29.8)	106 (25.3)	332 (26.8)
2008	75 (11.4)	22 (13.7)	56 (13.4)	153 (12.3)
Age, y, mean ± SD	35.6 ± 15.2	**32.8 ± 15.1†**	**29.7 ± 13.1‡**	33.2 ± 14.8
Female sex	236 (35.7)	54 (33.5)	144 (34.4)	434 (35.0)
Married or lived together	272 (41.2)	**48 (29.8)§**	**136 (32.5)§**	456 (36.7)
Unemployed, n = 1,239	257 (38.9)	62 (38.8)	155 (37.0)	474 (38.3)
Did not begin secondary level education, n = 1,235	150 (22.9)	31 (19.3)	**67 (16.0)§**	248 (20.1)
Tobacco use (ever), n = 1,240	191 (28.9)	48 (29.8)	**97 (23.2)†**	336 (27.1)
Alcohol use or abuse (ever), n = 1,240	257 (38.9)	61 (37.9)	148 (35.3)	466 (37.6)
Illicit drug use (ever)	131 (19.8)	38 (23.6)	77 (18.4)	246 (19.8)
Weight loss, n = 1,237	543 (82.5)	126 (78.8)	330 (78.8)	999 (80.8)
Dyspnea, n = 1,238	118 (17.9)	21 (13.1)	81 (19.3)	220 (17.8)
Hemoptysis, n = 1,239	28 (4.3)	9 (5.6)	**38 (9.1)§**	75 (6.1)
Cavitary lesion on chest radiography, n = 1,207	199 (16.5)	43 (3.6)	144 (11.9)	386 (32.0)
Low BMI, n = 1,233	203 (30.8)	55 (34.8)	133 (32.1)	391 (31.7)
Previous TB treatment	328 (49.6)	77 (47.8)	224 (53.5)	629 (50.7)
Type of TB				
Pulmonary only	650 (98.3)	155 (96.3)	414 (98.8)	1219 (98.2)
Extrapulmonary	11 (1.7)	6 (3.7)	5 (1.2)	22 (1.8)

*Values are no. (%) except as indicated. **Boldface** indicates significant difference in statistical comparison of baseline characteristics in the corresponding drug-resistance group to drug-susceptible cases. INH, isoniazid; RIF, rifampin; MDR TB, multidrug-resistant tuberculosis; DST, drug-susceptibility testing; BMI, body mass index.
†p<0.05.
‡p<0.001.
§p<0.01.

failure, 4 (22.2%) were adults with a household contact, and 1 (5.6%) had confirmed category I failure. Breslow-Day tests for homogeneity indicated that the effect of adult household contact on the odds of MDR TB is modified by diabetes ($p<0.0001$), and that the effect of suspected category I failure on the odds of MDR TB is modified by diabetes ($p = 0.0113$). One patient with new smear-positive TB was suspected of failing category II treatment (i.e., positive smear or culture during the second or third month of category II therapy); this same patient met the risk group criteria for adult household contact and private or self-administered treatment. Among previously treated patients with smear-positive TB, the following factors were significantly associated with a higher relative risk for MDR TB, compared with regional surveillance prevalence estimates: adult household contact (51.4%), failure of category I treatment (73.3%), early relapse after category I treatment (40.0%), suspected (84.6%) or confirmed (61.1%) failure of category II treatment, and history of ≥2 previous TB treatments (38.3%).

Discussion

We describe the proportion of drug resistance among TB patients as detected by using the screening strategy for MDR TB instituted in Lima, Peru, starting in 2005. When these data were compared with nearly concurrent population-based surveillance data, the proportion of MDR TB among new and previously treated TB cases was found to be significantly higher, indicating that screening high-risk patients may be an effective strategy. The proportion of MDR TB detected among patients with new smear-positive TB is comparable to that among previously treated patients with smear-positive TB in the cohort ($p = 0.458$). In the Peruvian NTP surveillance regional data corresponding to the study area, the prevalence of MDR TB among patients with new smear-positive TB was significantly lower than the prevalence among previously treated patients with smear-positive TB ($p<0.001$). This finding shows that the strategy implemented in Lima was especially effective in detecting MDR TB among patients with new smear-positive TB.

The risk groups with the highest rates of MDR TB were those with diabetes mellitus, adults or children with

Table 3. Prevalence estimates of TB drug resistance from national and regional surveillance data, and proportion of drug resistance in study cohort, Lima, Peru, 2005–2008*

Cohort	Total no. patients	No. (%) susceptible to INH and RIF	No. (%) monoresistant to INH or RIF	No. (%) MDR TB
Peruvian NTP drug-resistance surveillance, national data (*21*)				
All TB patients	2,167	1,829 (84.4)	158 (7.3)	180 (8.3)
New TB patients	1,816	1,597 (87.9)	124 (6.8)	95 (5.2)
Previously treated TB patients	351	232 (66.1)	34 (9.7)	85 (24.2)
Peruvian NTP drug-resistance surveillance, regional data corresponding to study area†				
All TB patients	580	467 (80.5)	41 (7.1)	72 (12.4)
New TB patients	476	396 (83.2)	33 (6.9)	47 (9.9)
Previously treated TB patients	104	71 (68.3)	8 (7.7)	25 (24.0)
Study cohort				
All TB patients	1,241	661 (53.3)	161 (13.0)	419 (33.8)
New TB patients	612	333 (54.4)	84 (13.7)	195 (31.9)
Previously treated TB patients	629	328 (52.2)	77 (12.2)	224 (35.6)
Study cohort, smear-positive samples only				
All TB patients	1,114	581 (52.2)	143 (12.8)	390 (35.0)
New TB patients	531	278 (52.4)	73 (13.8)	180 (33.9)
Previously treated TB patients	583	303 (52.0)	70 (12.0)	210 (36.0)

*TB, tuberculosis; INH, isoniazid; RIF, rifampin; MDR, multidrug resistant; NTP, National Tuberculosis Control Program.
†Ministerio de Salud, Peru, unpub. data.

household contacts with known or suspected TB, suspected failure of category I or II treatment (i.e., positive smear or culture during the second or third month of therapy), failure or early relapse to category I treatment, failure of category II treatment, and multiple (≥2) previous TB treatments. Ample literature supports these findings in a variety of settings (*22–25*). Screening for drug resistance among these groups is easily implemented and should be strongly considered by national TB programs.

In addition to identifying risk groups with high prevalence of MDR TB, other considerations are pertinent to the design of an optimal programmatic strategy. For example, risk groups with a relatively low prevalence of MDR TB may still merit DST if delays in initiation of MDR TB treatment would have severe consequences (e.g., children or HIV-positive patients) or if the absolute number of MDR TB cases within that risk group is substantial (e.g., patients with diabetes). The relative complexity of implementing certain testing strategies is also a consideration. Compared with alternative testing strategies such as universal testing or testing by geographic region, a strategy that focuses on high-risk patients requires training health care workers to screen each TB patient for numerous risk factors. Therefore, case finding may be variable under routine program conditions.

Table 4. MDR TB among new smear-positive TB patients compared with regional surveillance prevalence estimates, by NTP risk group, Lima, Peru, 2005–2008*

Risk factor	Total no. patients	No. (%) MDR TB	Odds ratio (95% CI)
New smear-positive TB patients in NTP regional surveillance data	476	47 (9.9)	
New smear-positive TB patients in study cohort	531	180 (33.9)	4.68 (3.30–6.65)
HIV positive	46	8 (17.4)	1.92 (0.85– 4.36)
Diabetes mellitus	107	18 (16.8)	1.85 (1.02–3.33)
Chronic corticosteroid therapy	4	0	NA
Other immunosuppression	5	0	NA
Adverse reaction	4	1 (25.0)	3.04 (0.06–38.63)
Previous hospitalization within the past 2 y with duration >15 d	5	2 (40.0)	6.09 (0.49–54.15)
Health care worker during the past 2 y	24	4 (16.7)	1.83 (0.60–5.57)
Health sciences student during the past 2 y	29	5 (17.2)	1.90 (0.69–5.22)
Prisoner during the past 2 y	27	4 (14.8)	1.59 (0.53–4.79)
Adult patient with household contact risk factor(s)†	170	57 (33.5)	4.60 (2.97–7.14)
Pediatric patient with household contact risk factor(s)†	13	7 (53.9)	10.65 (2.90–39.71)
Private or self-administered treatment	2	2 (100.0)	NA
Sputum positive during second or third month of category I treatment	105	70 (66.7)	18.26 (11.01–30.26)
Sputum positive during second or third month of category II treatment	1	1 (100.0)	NA

*MDR, multidrug resistant; TB, tuberculosis; NTP, National Tuberculosis Control Program; CI, confidence interval; NA, not applicable.
†Household contact risk factors are defined as household contact with a patient with known MDR TB, with a patient who showed TB treatment failure in the past 2 y, or with a patient being treated with second-line TB drugs.

Table 5. MDR TB among previously treated smear-positive TB patients compared with regional surveillance prevalence estimates, by NTP risk group, Lima, Peru, 2005–2008*

Risk factor	Total no. patients	No. (%) MDR TB	Odds ratio (95% CI)
Previously treated smear-positive TB patients in NTP regional surveillance data	104	25 (24.0)	
Previously treated smear-positive TB patients in study cohort	583	210 (36.0)	1.78 (1.10–2.88)
HIV positive	36	12 (33.3)	1.58 (0.69–3.61)
Diabetes mellitus	30	10 (33.3)	1.58 (0.65–3.82)
Adverse reaction	13	1 (7.7)	0.26 (0.01–1.97)
Previous hospitalization within the past 2 y with duration >15 d	4	1 (25.0)	1.05 (0.02–13.80)
Health care worker during the past 2 y	4	1 (25.0)	1.05 (0.02–13.80)
Health sciences student during the past 2 y	4	3 (75.0)	9.48 (0.71–503.7)
Prisoner during the past 2 y	24	4 (16.7)	0.63 (0.20–2.02)
Adult case with household contact risk factor(s)†	109	56 (51.4)	3.34 (1.86–6.00)
Pediatric case with household contact risk factor(s)†	7	3 (42.9)	2.37 (0.32–14.92)
Private or self-administered treatment	87	28 (32.2)	1.50 (0.79–2.83)
Sputum positive during second or third month of category I treatment	5	3 (60.0)	4.74 (0.50–58.69)
Sputum positive during second or third month of category II treatment	13	11 (84.6)	17.38 (3.36–166.8)
Failure of category I treatment‡	30	22 (73.3)	8.69 (3.44–21.93)
Relapsed within 6 mo after category I treatment§	65	26 (40.0)	2.11 (1.08–4.12)
Defaulted while receiving category I treatment¶	98	15 (15.3)	0.57 (0.28–1.16)
Failure of category II treatment‡	18	11 (61.1)	4.97 (1.74–14.18)
Relapsed within 6 mo after category II treatment§	8	5 (62.5)	5.27 (0.93–35.66)
Defaulted while receiving category II treatment¶	63	16 (25.4)	1.08 (0.52–2.22)
Chronic treatment (≥2 prior treatments)#	253	97 (38.3)	1.96 (1.17–3.29)

*MDR, multidrug resistant; TB, tuberculosis; CI, confidence interval; NTP, National Tuberculosis Control Program.
†Household contact risk factors are defined as household contact with a patient with known MDR TB, with a patient who showed TB treatment failure in the past 2 y, or with a patient being treated with second-line TB drugs.
‡Defined as positive smear and/or culture after >4 mo of treatment, or positive smear and/or culture upon finishing treatment.
§Defined as recurrence of disease <6 mo after being classified as cured by NTP norms.
¶Defined as not receiving treatment >1 mo upon enrollment into the study.
#Defined as a history of ≥2 previous TB treatments.

The findings of this evaluation are subject to several limitations. Although our study personnel would visit local health establishments in a purely observational capacity, the frequent visits by data collectors in the health centers could have sensitized health care workers to follow screening and referral protocols more closely than they would have otherwise. In addition, given the use of phenotypic methods, the drug-resistance status of particular isolates could be determined only for culture-positive samples. Although the yield of positive cultures was high for all methods used (67.4% for indirect conventional DST, 78.5% for direct NRA, and 35.3% for largely paucibacillary or smear-negative samples submitted for BACTEC; data not shown), the contribution to relative risk of MDR TB among those without DST results could not be determined. These results call attention to one of the shortcomings of all phenotypic methods, i.e., a substantial fraction of patients never have positive cultures or DST results to confirm the diagnosis or guide therapy, despite being at high risk for having MDR TB. Nonetheless, the yield of positive cultures in our sample is similar to that obtained by programs that have used the same sputum-processing methods (N-acetyl-L-cysteine and centrifugation, with cultivation on LJ medium). Finally, this evaluation was observational in nature and lacks a concurrent comparison group, such as one that had undergone an alternative screening strategy. On the other hand, this study has key strengths. The programmatic nature of this intervention, an active field presence to capture accurate and complete data on a large cohort, and the fortuitous concurrent surveillance study have allowed us to assess the effects of these programmatic efforts to identify patients with MDR TB.

To date, little research has been conducted on the comparative effectiveness of varied approaches to MDR TB screening and treatment referral. To our knowledge, the only study to show clinical results for performing rapid DST is a retrospective study carried out in California, which showed that using a molecular beacon assay led to earlier diagnosis and treatment initiation for MDR TB (*26*). An important aspect of program evaluation is feedback of findings to further improve treatment programs. In Peru, the results of this evaluation have been conveyed to the NTP and NRL. The aim of this communication is to describe an intensive evaluation of one of Peru's public health strategies for improving MDR TB control. In other low- to middle-income countries, similar program evaluations should be implemented to clarify national and regional MDR TB epidemiology, identify key risk groups for MDR TB, and inform national strategies to diagnose and treat MDR TB. Ultimately, the effects of these changes

on turn-around time, time to culture conversion, cure rates, and costs will determine the comparative success of these strategies.

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Staphylococcus aureus Infections in US Veterans, Maryland, USA, 1999–2008[1]

LaRee A. Tracy, Jon P. Furuno, Anthony D. Harris, Mary Singer, Patricia Langenberg, and Mary-Claire Roghmann

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- Describe the change in overall incidence of *S. aureus* infections between fiscal years 1999 and 2008 based on a retrospective cohort study using patient-level data in the Veterans Affairs Maryland Healthcare System
- Describe trends in invasive vs noninvasive *S. aureus* infections, changes in methicillin susceptibility, and changes in location of onset and infection site between fiscal years 1999 and 2008 based on the aforementioned study
- Describe hospital infection-control practices that may contribute to declining incidence of invasive *S. aureus* infections

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Trends in *Staphylococcus aureus* infections are not well described. To calculate incidence in overall *S. aureus* infection and invasive and noninvasive infections according to methicillin susceptibility and location, we conducted a 10-year population-based retrospective cohort study (1999–2008) using patient-level data in the Veterans Affairs Maryland Health Care System. We found 3,674 *S. aureus* infections: 2,816 (77%) were noninvasive; 2,256 (61%) were methicillin-resistant *S. aureus* (MRSA); 2,517 (69%) were community onset, and 1,157 (31%) were hospital onset. Sixty-one percent of noninvasive infections were skin and soft tissue infections; 1,112 (65%) of these were MRSA. Ten-year averaged incidence per 100,000 veterans was 749 (± 132 SD, range 549–954) overall, 178 (± 41 SD, range 114–259) invasive, and 571 (± 152 SD, range 364–801) noninvasive *S. aureus* infections. Incidence of all

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S. aureus infections significantly increased (p<0.001), driven by noninvasive, MRSA, and community-onset infections (p<0.001); incidence of invasive *S. aureus* infection significantly decreased (p<0.001).

Staphylococcus aureus exists as a commensal organism living on the human body in equilibrium with other bacteria and as a common agent associated with a spectrum of diseases ranging from mild, noninvasive skin and soft tissue infections (SSTIs) to invasive, life-threatening bloodstream infections. Increasing incidence of infections caused by methicillin-resistant *S. aureus* (MRSA) has complicated treatment of *S. aureus* infection. Previously MRSA infections were problematic primarily among hospitalized persons or persons exposed to the health care settings. However, since the 1990s, MRSA infections have become more prevalent in healthy, younger persons who have little to no exposure to health care settings. Of particular concern is the rapid increase in MRSA SSTIs reportedly driven by emergence of a new MRSA strain, USA300 (*1*,*2*).

Despite these changes, the epidemiology of *S. aureus* infection, particularly the total effect of infection in the United States, is not well described. Several population-based studies on *S. aureus* infections exist; however, these studies focused on hospital-based populations (*3*–*6*), MRSA infection (*7*–*9*), non-US populations (*10*–*12*), or only estimated the impact of invasive *S. aureus* disease (*10*,*13*–*15*). Additionally, population-level changes in incidence, particularly before and after USA300 MRSA emerged, are largely unknown. To describe overall trends and recent changes in the incidence of *S. aureus* infection while differentiating between invasive and noninvasive, community- and hospital-onset, and methicillin-susceptible and -resistant *S. aureus* infections, we conducted a retrospective population-based study.

Methods

Data Source

Our study used data from the Veterans Affairs Maryland Health Care System (VAMHCS) over a 10-year period (1999–2008). VAMHCS, a large, integrated health care system, comprises 3 medical centers (Baltimore VA Medical Center, Perry Point VA Medical Center, and the Baltimore VA Rehabilitation and Extended Care Center), ≈730 inpatient beds, and 5 community-based outpatient clinics. VAMHCS uses an electronic health information system known as the Veterans Health Information Systems and Technology Architecture (VistA). This system is used to collect and maintain all health information at each VA medical facility, including the VAMHCS. VAMHCS's electronic medical records and administrative data, including codes from the International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM), and microbiology culture results collected from the VistA served as the primary data source for this study. The VAMHCS Research and Development Committee and the University of Maryland, Baltimore Institutional Review Board approved this study. Given that data were retrospectively obtained from VistA, informed consent requirements were waived.

S. *aureus* Culture Collection and Classification

We collected data during October 1, 1998–September 30, 2008, on all *S. aureus*–positive blood and clinical cultures (excluding surveillance and fecal and nasal cultures) identified through microbiologic accession number, date, time, and specimen type (e.g., blood, skin). Each positive culture was classified as originating from a sterile or a nonsterile body site. We defined a sterile body site according to Centers for Disease Control and Prevention Active Bacterial Core surveillance criteria, and all other body sites were classified as nonsterile (*13*). We defined a unique culture as the first *S. aureus*–positive culture obtained from a patient during a 6-month period. If cultures were obtained from a sterile and a nonsterile site from the patient during the same period, we chose the culture from the sterile site.

For *S. aureus*–positive cultures obtained during an outpatient visit for which the patient was not subsequently hospitalized within a 72-hour period after culture, we obtained all ICD-9-CM codes associated with all of the patient's outpatient visits on the day of culture. For *S. aureus*–positive cultures obtained during an outpatient visit for which the patient was subsequently hospitalized within a 72-hour period after culture, we collected the outpatient and hospital discharge ICD-9-CM codes. Finally, we obtained all ICD-9-CM discharge codes for *S. aureus*–positive cultures obtained during a hospitalization. Using information from previous studies, we developed a comprehensive list of ICD-9-CM codes for *S. aureus*–related infections and categorized them by the site of infection most consistent with the associated code (*16*).

We determined an invasive *S. aureus* infection on the basis of *S. aureus* isolation from a clinical or blood culture from a normally sterile body site, such as blood, cerebrospinal fluid, pleural fluid, pericardial fluid, peritoneal fluid, joint/synovial fluid, bone, internal body site, and muscle. Identification of noninvasive *S. aureus* infection was based on isolation of *S. aureus* from a clinical culture of a nonsterile site, without a concurrent culture from a sterile site obtained during the same 6-month period, and at least 1 ICD-9-CM code for *S. aureus*–related infection from the outpatient visit or hospitalization associated with

the positive culture. For a noninvasive infection, we based the requirement of a matching ICD-9-CM code along with positive culture on concerns that *S. aureus* obtained from a sample from a nonsterile body site can represent either infection or colonization. In a substudy that used a random sample of cases, we estimated that an ICD-9-CM code for *S. aureus*–related infection plus positive clinical culture (from a nonsterile site) increases the probability of a true noninvasive *S. aureus* infection by ≈23.8% over positive clinical culture alone (*16*).

Positive cultures obtained after the first 48 hours of hospitalization, rehabilitation, or long-term stay were classified as hospital onset and all others as community onset. All *S. aureus* infections were classified according to methicillin susceptibility on the basis of in vitro susceptibility to oxacillin. All MRSA infections were grouped into the following epidemiologic categories: health care–associated community onset, defined as cases in persons with at least 1 listed risk factor in the past 12 months; health care–associated hospital onset, defined as cases in persons who have a positive culture within 48 hours after hospitalization; or community-associated, defined as cases in persons with no documented health care–associated community-onset risk factor (*13*). To determine a risk factor for health care–associated community-onset infection, we obtained history of hospitalization, surgery, residence in a long-term care facility, or prior MRSA-positive culture in the past 12 months before the date and time of each index positive MRSA culture. Site of infection (bone or joint, skin or soft tissue, endovascular, respiratory, intraabdominal/pelvic, central nervous system, urinary tract, *S. aureus*–nonspecific site, bacteremia without focus, and other or site not specified) was determined for each *S. aureus* infection on the basis of matching ICD-9-CM code. When cultures matched with multiple ICD-9-CM codes, we chose the highest ranking site of infection on the basis of the likelihood that a culture represented true *S. aureus* infection (*16*).

Statistical Analysis

Information about annual number of unique veterans, admissions, and total inpatient days for hospitalization, long-term care, and residential rehabilitation programs were obtained from the VAMHCS Medical Administrative Service fiscal year (FY) databases. Annual and 10-year averaged incidence rates per 100,000 veterans were estimated overall and for community-onset infections and per 100,000 inpatient days for hospital-onset *S. aureus* infections. To account for the first 48 hours in the definition for hospital-onset infection, we adjusted inpatient days (number of annual inpatient days minus 2× the number of annual admissions) (*13*). Because the average inpatient length of stay in the VAMHCS is >48 hours, this adjustment provides a consistent and reasonably accurate estimate of inpatient days past 48 hours.

Trends in all *S. aureus* infections were initially assessed by plotting natural and cubic spline smoothers to the observed data plotted as a function of time. The formal analysis of trends of all *S. aureus* infections was based on generalized linear models, assuming a Poisson distribution with a log link function, including FY as a predictor variable and log total number of unique veterans or log-adjusted inpatient days as an offset variable (*17*). Model fit was assessed by evaluating the deviance and Akaike information criterion, and regression coefficients for trend were assessed by the partial Wald test (*18*,*19*). Additional models were fit for each stratum of interest, i.e., invasive, noninvasive, onset, and methicillin susceptibility. Analyses were performed by using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) and R version 2.7.0 (2008) software (www.r-project.org).

Results

For FY 1999–FY 2008, a mean (± SD) of 48,940 (± 3,926) unique veterans accessed care in the VAMHCS each year. The mean annual numbers of acute care and nursing home or intermediate care admissions was 5,854 (± 199) and 919 (± 161) corresponding to 23,183 (± 1,743) and 98,902 (± 10,893) inpatient days, respectively.

Overall Incidence of *S. aureus* Infection

We identified 3,674 *S. aureus* infections, of which 2,816 (77%) were noninvasive and 2,256 (61%) were MRSA. The overall proportion of community-onset and hospital-onset infections was 2,517 (69%) and 1,157 (31%), respectively. The 10-year averaged incidence per 100,000 veterans was 749 cases (± 132 SD, range 549–954) overall, 178 (± 41 SD, range 114–259) invasive, and 571 (± 152 SD, range 364–801) for noninvasive *S. aureus* infections. The annual incidence per 100,000 veterans of all *S. aureus* infections increased significantly starting in 2003 (p<0.001). This increase was driven by significant (p<0.001) increases in noninvasive, MRSA, and community-onset infections (Figure 1).

Invasive *S. aureus* Infections

We identified 858 invasive *S. aureus* infections, of which 75% were based on positive blood cultures, among 800 unique veterans during FY 1999–FY 2008. The proportions of community- and hospital-onset invasive *S. aureus* infections were 56% and 44%, respectively; 52% were caused by MRSA (Table 1). Among all 449 invasive MRSA infections, 243 (54%) were epidemiologically classified as health care–associated hospital-onset, 152 (34%) as health care–associated community-onset, and 54 (12%) as community-associated MRSA.

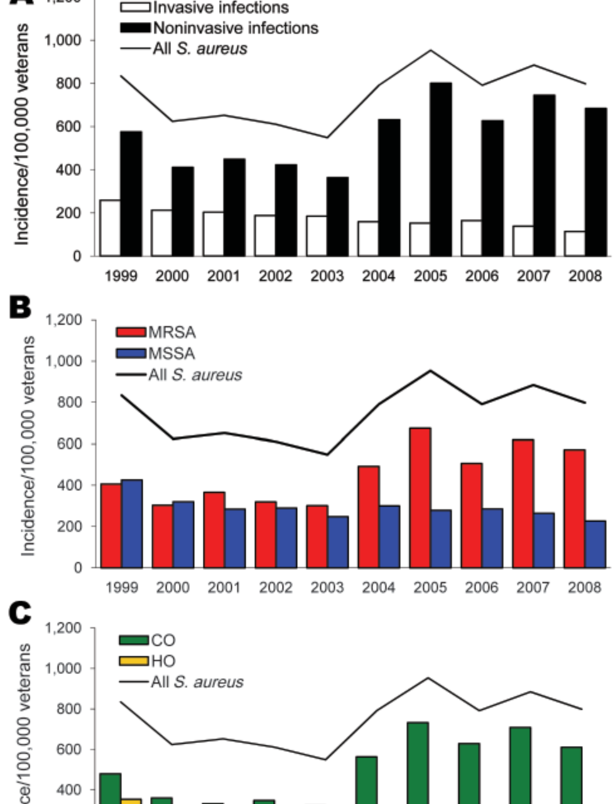

Figure 1. Incidence per 100,000 veterans of *Staphylococcus aureus* infections by invasive and noninvasive (A), methicillin susceptibility (B), and onset (C), Veterans Affairs Maryland Health Care System, fiscal years 1999–2008. Solid line represents all *S. aureus* infections. MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; CO, community onset; HO, hospital onset.

The annual incidence of all invasive *S. aureus* infections decreased gradually from FY 1999 through FY 2008 (Figure 1, panel A). In FY 1999, the estimated incidence of invasive *S. aureus* infection was 259 infections per 100,000 veterans; however, by FY 2008, incidence was 114 per 100,000 veterans (p<0.001). This decrease appears to be associated with overall decreases in the incidence of invasive MRSA (135–68/100,000; p = 0.02) and methicillin-susceptible *S. aureus* (MSSA) (123–47/100,000; p = 0.009).

The incidence of invasive hospital-onset *S. aureus* infections decreased from 150 to 85 cases per 100,000 adjusted acute-care inpatient days (Table 2). Invasive hospital-onset MSSA and MRSA infections significantly decreased during the 10-year period (p = 0.01 and p<0.001, respectively). The incidence of invasive community-onset *S. aureus* infections decreased 1.9-fold from 119 to 64 per 100,000 veterans and was driven by decreases in incidence of invasive community-onset MSSA. Incidence of invasive community-onset MRSA remained relatively unchanged (46 to 41/100,000 veterans).

Noninvasive *S. aureus* Infections

We identified 2,816 noninvasive *S. aureus* infections among 2,511 unique patients during FY 1999–FY 2008. Overall, 28% and 72% of *S. aureus* infections were noninvasive hospital onset and community onset, respectively; 1,807 (64%) were caused by MRSA and 1,006 (36%) by MSSA (Table 1). Of MRSA infections, 539 (30%), 572 (32%), and 696 (39%) were epidemiologically classified as health care–associated community-onset, health care–associated community-onset, and community-associated MRSA infections, respectively.

From FY 1999 through FY 2008, the overall incidence of noninvasive *S. aureus* infections increased significantly (p<0.001) (Figure 1, panel A). Incidence in FY 1999 was 576 cases per 100,000 veterans and rapidly increased beginning in FY 2003, peaking at 801 cases per 100,000 veterans by FY 2005. These increases were driven primarily by increases in noninvasive MRSA infections, with the most pronounced increases occurring during FY 2003 and FY 2004 (Table 2). The overall incidence of health care–associated hospital-onset MRSA infections decreased nonsignificantly (p = 0.47); however, health care–associated community-onset and community-associated MRSA significantly increased (p<0.001), particularly after FY 2003.

The incidence of noninvasive hospital-onset infections did not follow any apparent increasing or decreasing trend (p = 0.08; Table 2). However, driven by noninvasive MRSA infections, the overall incidence of noninvasive community-onset *S. aureus* infections increased significantly (p<0.001). Incidence after FY 2003 rapidly increased from 218 to 546, peaking at 644 cases per 100,000 veterans in FY 2005. After FY 2000, incidence of noninvasive community-onset MRSA infections increased 4-fold from 100 to 397 cases per 100,000 veterans. Incidence of noninvasive community-onset MSSA infections did not change (p = 0.83; Table 2).

A total of 1,703 (61%) noninvasive infections were classified as SSTIs of which 1,112 (65%) were caused by MRSA. Changes in incidence of overall noninvasive MRSA infections were driven by increases in MRSA SSTIs (Figure 2). Incidence per 100,000 veterans significantly increased from 90 cases in FY 1999 to 345 in FY 2008 (p<0.0001); the largest increase began in FY 2003 and incidence peaked at 440 cases in FY 2005.

Discussion

We have described the incidence of all *S. aureus* infections during a 10-year period in a large US-based

Table 1. Characteristics of *Staphylococcus aureus* infections, Veterans Affairs Maryland Health Care System, fiscal years 1999–2008*

Characteristic	Invasive *S. aureus* infections					Noninvasive *S. aureus* infections				
		Onset		Susceptibility			Onset		Susceptibility†	
	Total, n = 858	H, n = 381	C, n = 477	MRSA, n = 449	MSSA, n = 409	Total, n = 2,816	H, n = 778	C, n = 2,038	MRSA, n = 1,807	MSSA, n = 1,006
No. patients	800	359	441	415	385	2,511	708	1,803	1,600	908
Age, y‡										
Mean (SD)	64 (14)	67 (13)	61 (13)	65 (13)	62 (14)	62 (14)	68 (13)	60 (14)	62 (14)	62 (14)
Median	64	70	60	65	61	61	71	58	61	60
Range	28–98	28–98	30–94	28–97	29–98	19–94	19–93	22–94	23–94	19–93
Male sex, no. (%)	782 (98)	370 (97)	469 (98)	399 (98)	440 (98)	2,436 (97)	691 (98)	1,745 (97)	1,552 (97)	881 (97)
Race, no. (%)										
Black	393 (49)	154 (43)	239 (54)	190 (48)	203 (53)	1,212 (48)	289 (41)	923 (51)	781 (49)	429 (47)
White	387 (48)	200 (56)	187 (42)	217 (52)	170 (44)	1,186 (47)	412 (58)	774 (43)	745 (47)	440 (49)
Other§	20 (3)	5 (1)	15 (3)	8 (2)	12 (3)	113 (5)	7 (1)	106 (6)	74 (5)	39 (4)

*MRSA, methicillin-resistant *S. aureus*. MSSA, methicillin-susceptible *S. aureus*; H, hospital; C, community.
†Methicillin-susceptibility unknown for 3 patients with noninvasive *S. aureus* infections.
‡Age unknown for 8 patients with noninvasive *S. aureus* infections.
§Includes missing data. Other/unknown race not included in test.

population (49,000 persons) using person-level data including clinical culture and administrative data to identify and classify infections. Our results suggest significant increases in overall *S. aureus* infections from FY 1999 through FY 2008; the largest increases were associated with community-onset MRSA infections of skin and soft tissue and an overall decrease in incidence of invasive *S. aureus* infections.

During the study period, we implemented many new hospital infection–control practices—including the use of alcohol-based hand gels for hand hygiene (2003), central line bundles (2006), MRSA surveillance cultures (intensive care units in 2003, expanded to acute care in 2007), and chlorhexidine bathing of all surgical patients (2009)—which may have contributed to the decreased incidence of invasive infections. Previous studies suggest that improved infection control practices have contributed to fewer catheter-related and central line–associated bloodstream infections (*20*,*21*). However, attributing the decrease to a single practice is difficult, if not impossible, and few published studies exist with which we can compare our invasive *S. aureus* results.

Laupland et al. estimated an annual incidence of invasive *S. aureus* of 28 cases per 100,000 population, but their results may not be comparable because they were based primarily on MSSA infections (*12*). Klevens et al. estimated an incidence of invasive MRSA infection of 32 cases per 100,000 population, 75% were bacteremias, and 27% were hospital-acquired on the basis of 2005 data from 9 US cities (*13*). Their study reported a noticeably higher incidence of invasive MRSA in Baltimore, Maryland, USA, of 117 per 100,000 compared with estimates of 20–50 per 100,000 population at other sites. Our estimated rate of invasive MRSA infection (160 cases/100,000 veterans) is commensurate during the same year (2005), and our calculated proportion of invasive hospital-onset MRSA

Table 2. Incidence and type of *Staphylococcus aureus* infections, Veterans Affairs Maryland Health Care System, fiscal years 1999–2008*

Fiscal year	Invasive infection				Noninvasive infection			
	Hospital onset†		Community onset‡		Hospital onset†		Community onset‡	
	MSSA	MRSA	MSSA	MRSA	MSSA	MRSA	MSSA	MRSA
1999	46.3	104.1	72.6	46.0	88.7	250.7	246.7	113.7
2000	58.4	75.1	72.4	44.4	116.8	183.5	142.5	100.5
2001	50.6	109.6	66.3	27.8	139.1	273.9	117.6	119.7
2002	58.1	116.1	57.5	31.7	111.7	254.6	150.8	107.1
2003	53.2	87.1	59.0	43.3	135.5	222.6	104.2	114.0
2004	24.3	72.8	59.2	51.3	140.8	301.0	165.8	286.3
2005	33.5	54.4	47.1	41.2	62.8	272.1	176.6	467.0
2006	59.3	59.3	57.5	46.0	91.2	150.4	164.8	360.2
2007	29.3	37.7	46.3	48.3	87.9	196.8	162.2	451.7
2008	40.3	44.3	23.3	40.7	64.4	221.5	149.2	397.2
Average	45.3	76.0	56.1	42.1	103.9	232.7	158.0	251.8

*MSSA, methicillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*.
†Incidence per 100,000 adjusted acute-care inpatient days.
‡Incidence per 100,000 veterans.

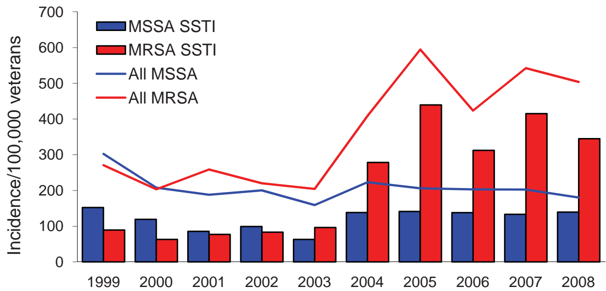

Figure 2. Incidence per 100,000 veterans of skin and soft tissue infections (SSTIs) caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA), Veterans Affairs Maryland Health Care System, fiscal years 1999–2008.

in 2005 (20%) also was similar. Klevens et al. reported estimates per 100,000 persons; therefore a direct comparison of rates of invasive hospital-onset MRSA infection is not feasible. However, our rates are potentially higher given the inpatient days adjustment accounting for the first 48 hours in the definition for hospital-onset infection. Another study by Laupland et al. reported an annual incidence of *S. aureus* bloodstream infections during 2000–2006 of 20 per 100,000 population (*14*). However, few MRSA cases were identified in this study, thereby making it difficult to compare with our results for which MRSA caused almost half of infections.

For several reasons, our incidence rates of invasive infections are higher than those previously reported. Black race has previously been reported as a marker for increased risk for invasive MRSA infections (*13*,*22*). Therefore, we would expect a higher incidence of all *S. aureus* infections, given that in our study 49% of patients were black. Additionally, given that our study was performed on data from a primarily urban population located in or around Baltimore, we could attribute the higher incidence of infections to suspected risk factors that are more prevalent in this location, including intravenous drug use.

Our study identified increases in noninvasive *S. aureus* infections, particularly around 2003, which most likely are associated with the emergence of the USA300 MRSA clone that has led to increases in community-associated MRSA, specifically in SSTIs (*1*,*22*). Also, despite dramatic increases in noninvasive community-onset MRSA infections, we did not observe a proportionate increase in invasive community-onset MRSA as might be expected if USA300 MRSA had the same propensity as non-USA300 MRSA to invade the bloodstream. No population-based studies have been published with which to compare overall noninvasive *S. aureus* infections, and few exist for comparison of noninvasive MRSA infections. For instance, Liu et al. recently reported annual (2004–2005) incidence rates of community-acquired and hospital-acquired MRSA among residents in San Francisco, California, USA, community-associated of 316 and 31 cases per 100,000 population, respectively, for which most cultures were from skin and soft tissue (*8*). The results of our study for community-onset MRSA are slightly lower but similar. Crum et al. reported a dramatic increase during 2002–2004 in community-associated MRSA infections, of which most were classified as SSTIs, and an incidence rate of 155 cases per 100,000 persons from 2004 data (*7*). The FY 2004 incidence of noninvasive community-associated MRSA in our study was 188 cases per 100,000 veterans, which is similar to that reported by Crum et al.

Our study adds new information to the existing literature and has several strengths. A major strength is its calculation of annual incidence of all *S. aureus* infections for a 10-year period by using actual numbers of patients at risk, admissions, and inpatient days. Our estimates of incidence are more accurate than those in previous studies, which were based on census-level data (*11*–*13*,*23*). Noninvasive infections were identified by using an automated approach that required both positive clinical culture and confirmed ICD-9-CM code for infection. This definition is more rigorous, thereby producing higher positive predictive values than clinical cultures alone, particularly for infections of bone and skin or soft tissue (*16*). In addition, this automated approach enabled us to identify and classify types of *S. aureus* infection, which is useful for understanding the overall population distribution of infection. Access to comprehensive, patient-level information, including prior hospitalizations, prior MRSA infections, and surgeries, allowed us determine the epidemiologic class for all MRSA infections. This study was performed in a population receiving standardized health care; therefore, findings should be free of bias associated with access to care or duration and type of treatment received.

Our study also has several limitations. First, the VAMHCS population of adult, mostly male patients living in the mid-Atlantic region does not fully represent the overall US population. We are unable to extrapolate these findings to children, a population for which an increase of community-onset MRSA SSTIs has been reported (*24*). Previous reports suggest that men are at higher risk than women for *S. aureus* infections; therefore, our estimates may overestimate true rates for women (*8*,*12*,*13*). Second, although we did not perform molecular typing on the *S. aureus*–positive isolates, we expect that a significant proportion (>80%) of noninvasive MRSA infections were caused by the USA300 MRSA strain (*1*). Given that USA300 MRSA reportedly varies across the United States, our findings may not be generalizable to populations in which MRSA strains differ. Third, we may have underestimated

incidence because our definition of *S. aureus* infection required a positive clinical or blood culture. However, given the standardized access to care in the VAMHCS, we expect that cultures were uniformly collected in patients who had clinical signs or symptoms of infection. Fourth, the clinical culturing rate may have increased during our study period, which would contribute to overestimates in the incidence of *S. aureus* infection, particularly noninvasive infections. Johnston et al. observed an increase in the absolute number of SSTI cultures obtained in the VAMHCS Emergency Care Service (*1*). However, they determined that the proportion of MRSA infections increased, even though the proportion of MSSA remained the same, which suggests a true increase in MRSA. We observed similar patterns: whereas MRSA infections increased, MSSA infections remained relatively stable, and the annual number of *S. aureus* cultures did not significantly change.

In conclusion, this large, population-based study demonstrated an increase in the overall incidence of *S. aureus* infections during FY 1999–FY 2008, which was driven by a rapid increase in noninvasive, community-onset, MRSA skin and soft tissue infections. This increase was most striking during and after 2003, which is coincident with the time during which the USA300 clone became a major contributor to noninvasive *S. aureus* infections. Despite this increase, incidence of invasive community-onset MRSA infections did not significantly increase, and the overall MSSA infections and noninvasive MSSA infections remained generally stable. These results suggest a shift in the distribution of *S. aureus* infections to more noninvasive community-onset MRSA infections. This information is useful for interpreting changes in the epidemiology of *S. aureus* infections, which may help guide additional prevention strategies focused on reducing community-onset *S. aureus* infections. To further understand these trends, additional studies are warranted to identify risk factors for *S. aureus* infection and to describe the epidemiology of *S. aureus* infections across the entire population.

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Dr Tracy is an epidemiologist and statistician at the University of Maryland, Baltimore, School of Medicine, Department of Epidemiology and Public Health. This study was part of her doctoral research in epidemiology. Her main research interests are epidemiologic patterns and mathematical modeling of infectious diseases, particularly of MRSA infections.

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Amplification of Emerging Viruses in a Bat Colony

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Bats host noteworthy viral pathogens, including coronaviruses, astroviruses, and adenoviruses. Knowledge on the ecology of reservoir-borne viruses is critical for preventive approaches against zoonotic epidemics. We studied a maternity colony of *Myotis myotis* bats in the attic of a private house in a suburban neighborhood in Rhineland-Palatinate, Germany, during 2008, 2009, and 2010. One coronavirus, 6 astroviruses, and 1 novel adenovirus were identified and monitored quantitatively. Strong and specific amplification of RNA viruses, but not of DNA viruses, occurred during colony formation and after parturition. The breeding success of the colony was significantly better in 2010 than in 2008, in spite of stronger amplification of coronaviruses and astroviruses in 2010, suggesting that these viruses had little pathogenic influence on bats. However, the general correlation of virus and bat population dynamics suggests that bats control infections similar to other mammals and that they may well experience epidemics of viruses under certain circumstances.

Bats (Chiroptera) constitute ≈20% of living mammal species and are distributed on all continents except Antarctica (*1*). Their ability to fly and migrate, as well as the large sizes of social groups, predispose them for the acquisition and maintenance of viruses (*2*). Although the ways of contact are unknown, bat-borne viruses can be passed to other mammals and cause epidemics (*2*,*3*). Several seminal studies have recently implicated bats as sources of important RNA viruses of humans and livestock, including lyssaviruses, coronaviruses (CoVs), filoviruses, henipaviruses, and astroviruses (AstVs) (*2*,*4*). DNA viruses, including herpesviruses and adenoviruses (AdVs), have also been detected in bats, although with less clear implications regarding the role of bats as sources of infection for other mammals (*5*–*8*). While most of the above-mentioned viruses are carried by tropical fruit bats (Megachiroptera), the predominant hosts of mammalian CoVs, including those related to the agent of severe acute respiratory syndrome (SARS), are insectivorous bats (Microchiroptera) that are not restricted to tropical climates (*1*). By demonstrating the presence of SARS-related CoV in Europe, we have recently shown that the geographic extent of its reservoir is much larger than that of other bat-borne viruses, including Ebola, Marburg, Nipah, and Hendra (*9*).

In spite of the potential for serious consequences of virus epidemics emerging from bats, knowledge is currently lacking on the ecology of bat-borne viruses in bat reservoirs. We do not know how viruses with human pathogenic potential are maintained in bat populations, whether and how they are amplified and controlled, and whether they cause effects on individual bats or on bat populations. The current lack of data is due to difficulties in monitoring virus populations (rather than bat populations) in sufficient density. Available studies have focused on lyssaviruses, hennipaviruses, and filoviruses, which have extremely low detection frequencies, thus causing viruses to be encountered too rarely to enable the characterization of virus frequency and concentration over time (*10*–*15*). These studies have therefore relied on antibody testing, which provides higher detection rates by making indirect and cumulative assessments of virus contact during the

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lifetime of bats (*10–15*). However, results of antibody testing fail to correlate with the current presence of virus, preventing reliable analysis of a time component.

In a recent study, we obtained preliminary statistical hints that bats were more likely to carry CoV if they were young (*16*). In adult bats, a significant risk of carrying virus was identified for lactating females (*16*). Taking these clues together, we speculated that maternity roosts, inhabited predominantly by lactating females and newborns, with few adult males (*17*), might serve as the compartment of CoV amplification within the yearly life-cycle of bats in temperate climates. We therefore investigated the patterns of maintenance and amplification of specific RNA- and DNA viruses by direct and quantitative virus detection in a maternity colony over 3 consecutive years. RNA- and DNA viruses were examined because of their different abilities to persist and to rapidly generate new variants. Viruses identified included 1 CoV, 6 different AstVs, as well as a novel bat AdV. To assess the pathogenic influence of these viruses on bats, we quantified the reproductive success of the colony over the same time period.

Materials and Methods

Sample Collection and Preparation

Permission for this work on protected bats was obtained from the environmental protection authority (Struktur-Und Genehmigungsbehörde Nord Koblenz) of the German federal state of Rhineland-Palatinate. Sampling took place over 3 consecutive years: 2008, 2009, and 2010. The sampling site was the attic of a private house in a suburban area in the state of Rhineland-Palatinate, western Germany (Figure 1). The study did not involve any direct manipulations of bats and relied entirely on collection of fecal samples from the attic floor. Classification of bats as *Myotis myotis* was confirmed by mitochondrial DNA typing as described (*9*). Adult female bats leaving the roost were counted by trained field biologists before and after parturition. Pups were counted in the sampling site after the departure of adults. For each sampling date, plastic film was spread in the evening on the ground of a 20-m^2 attic compartment, and fresh droppings were collected with clean disposable forks the following night. Each sample consisted of exactly 5 fecal pellets collected in proximity and added to RNAlater RNA preservative solution (QIAGEN, Hilden, Germany). The equivalent of ≈100 mg was purified by the Viral RNA kit (QIAGEN) according to manufacturer's instructions.

Detection and Quantification of Viral RNA/DNA

Five microliters of RNA/DNA eluate were tested by broad range reverse transcription–PCR (RT-PCR) assays for the whole subfamily *Coronavirinae* (*16*), the family *Astroviridae* (*4*), and the genus *Mastadenovirus* (*18*). Specific real-time RT-PCR oligonucleotides were designed within the initial PCR fragments (those used are shown in Table 1). All 4 described real-time RT-PCR assays showed comparable lower limits of detection in the single copy range. Twenty-five–microliter reactions used the SuperScript III PlatinumOne-Step qRT-PCR Kit (Invitrogen, Karlsruhe, Germany) for detecting CoVs and AstVs in *M. myotis* bats or the Platinum Taq DNA Polymerase Kit (Invitrogen) for

Figure 1. A) Location of studied maternity bat roost (indicated by asterisk) in the state of Rhineland-Palatinate, Germany (50°25′46.91″N, 6°55′52.17″E). Red shading indicates the distribution of the studied bat species (adapted from the IUCN Red List of Threatened Species, v. 2010; www.iucnredlist.org). B) Cluster of *Myotis myotis* female bats hanging from the roof interior.

Table 1. Real-time reverse transcription–PCR oligonucleotides used for RNA virus testing, Germany, 2008–2010*

Virus targeted	Oligonucleotide ID	Sequence, 5′ → 3′	Orientation
Coronavirus	CoV-F	CGTCTGGTGATGCTACTACTGCTT	+
	CoV-P	FAM-TGCAAATTCCGTCTTTAAT-MGBNFQ	Probe
	CoV-R	CATTGGCACTAACAGCCTGAAA	–
Astrovirus	AstVa-F	GCTTGATCCWGTCTATCATACTGATG	+
	AstVa-P	FAM-CTTTTGAGTTTGCGTATGTTCA-MGBNFQ	Probe
	AstVa-R	CACATTTTTTCCATTCTTCTTCAAG	–
	AstVb-F	TATGTACTACTGCCTTCTGGTGAAATC	+
	AstVb-P	YAK-CCCACCAAACTCGCGGGAATCCT-BBQ1	Probe
	AstVb-R	TTATCCATCGTTGTGCTCACTTG	–
Adenovirus	AdV-F	GCGGTTGCAGCTAAGATTTGT	+
	AdV-P	FAM-CCCGTGGACAAAGAAGACACCCAGTATG-BBQ1	Probe
	AdV-R	CCAGCTGGAAGCGTGTTTTAT	–

*ID, identification; CoV, coronavirus; AstV, atrovirus; AdV, adenovirus; FAM, 6-carboxyfluorescein; MGB, minor groove binder; NFQ, nonfluorescent quencher; YAK, Yakima yellow; BBQ, black berry quencher; +, positive; –, negative.

detecting AdVs in *M. myotis* bats. Reactions were generally composed as follows: 400 nmol/L of the respective primers, 200 nmol/L of the respective hydrolysis probe, 0.5 μL enzyme mix or 0.1 μL Platinum Taq, 1 μg bovine serum albumin, and 5 μL RNA/DNA extract. For AdV DNA PCR, supplements of 0.2 mmol/L of each dNTP and 2.0 mmol/L of MgCL were added. Amplification involved 15 min at 55°C for reverse transcription of RNA viruses and 3 min at 95°C, followed by 45 cycles of 15 seconds at 94°C, and 25 seconds at 58°C for all viruses. Fluorescence was measured at the 58°C annealing/extension step.

For quantification, PCR amplicons from the initial screening assay were TA cloned in a pCR 4.0 vector (Invitrogen). Plasmids were then purified and reamplified with vector-specific oligonucleotides, followed by in vitro transcription with a T7 promotor-based Megascript kit (Applied Biosystems, Darmstadt, Germany). The in vitro–transcribed RNAs or, in the case of AdVs, the photometrically quantified plasmid alone, were used as calibration standards for virus quantification in bat fecal samples, as described previously (*19*).

In Silico Analyses

Sanger sequencing of PCR products was done by using dye terminator chemistry (Applied Biosystems). Nucleic acid alignments with prototype virus sequences were done based on amino acid code by the BLOSUM algorithm in the MEGA4 software package (www.megasoftware.net). Neighbor-joining phylogenies used an amino-acid percentage distance substitution model and 1,000 bootstrap reiterations. All sequences were submitted to GenBank under accession nos. HM368166–HM368175. All analyses were performed with Epi Info 3.5.1 (www.cdc.gov/epiinfo) and with SPSS 17 (SPSS, Munich, Germany).

Results

In a first step, the *M. myotis* maternity colony was surveyed for bat-borne RNA viruses. Broad-range RT-PCR assays for CoVs and AstVs were employed on samples taken in 2008. Screening was extended to include AdVs described in microchiroptera and megachiroptera bats (*6,8,20*). As shown in Figure 2, a CoV, 6 different AstVs, and 1 novel AdV were found. The CoV (GenBank accession no. HM368166) was a member of the genus *Alphacoronavirus* and belonged to a tentative species defined by bat-CoV HKU6 (97.4% amino acid identity in RNA-dependent RNA polymerase [RdRp], typing criteria as defined in [*9*]). The 6 different mamastroviruses (GenBank accession nos. HM368168–HM368175) clustered phylogenetically with bat-associated AstV, which has been described previously (*4,21*), showing 65.0%–86.0% amino acid identities with related bat-associated AstV from *M. chinensis* and *M. ricketti* bats from the People's Republic of China (Figure 2). The AdV constituted a novel *Mastadenovirus* species (GenBank accession no. HM368167) that was clearly separated from a clade of AdV recently reported in a *M. ricketti* bat in China and a *Pipistrellus pipistrellus* bat in Germany (*6,20*) (A. Kurth, pers. comm.). The closest relatives were bovine AdV C10 (GenBank accession no. AF282774) and Tupaia AdV (GenBank accession no. NC_004453), with 90.0% and 91.0% identity on the amino acid level, respectively. Amino acid identity with the Chinese bat AdV TJM (GenBank accession no. GU226970) was 83.5%.

For all 3 viruses, strain-specific real-time RT-PCR assays, including cloned, in vitro–transcribed RNA or plasmid DNA quantification standards, were generated (Table 1). For AstV, 2 assays had to be designed to cover the high diversity of AstVs that was found. These assays were used to monitor virus abundance in the *M. myotis* bat maternity colony over time. Populating of the roost started in March 2008. Sampling started in the second week of May when the colony reached full size. Sampling extended over 5 sampling dates until late July 2008 (Figure 3); 195 pooled samples, equal to 975 fecal pellets, were collected during this time. As shown in Figure

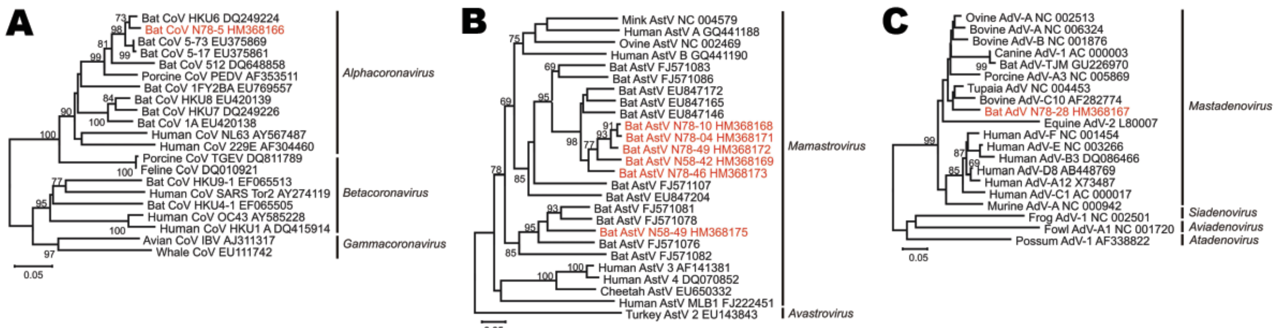

Figure 2. Phylogenetic relationships of novel bat viruses. A) Coronavirus, B) astrovirus, C) adenovirus. Neighbor-joining phylogenies were generated with MEGA (www.megasoftware.net), by using an amino acid percentage distance substitution model drawn to scale, complete deletion option, and 1,000 bootstrap reiterations. Bootstrap values are shown next to the branches; values <65 were removed for graphic reasons. Viruses newly identified in this study are shown in red. Viral genera are depicted next to taxon names. The BLOSUM aligned datasets corresponded to an 816-nt alignment, corresponding to nucleotides 14,781–15,596 in severe acute respiratory syndrome coronavirus (SARS-CoV) strain Tor2 (GenBank accession no. AY274119) for CoVs (A); a 381-nt alignment corresponding to nt 3,437–3,817 in mink astrovirus (AstV) (GenBank NC_004579) for AstVs (B); and to a 255-nt alignment corresponding to nt 46–300 in the bovine adenovirus (AdV) C10 hexon gene (GenBank accession no. AF282774) for AdVs (C). Trees were visualized in MEGA4, with prototype virus sequences restricted to ≈20 taxa additional to newly identified viruses for graphic reasons. Scale bars indicate amino acid substitutions per site.

3, panel A, 2 peaks of amplification of CoV occurred, characterized by increased virus concentrations and increased detection rates. The first peak was observed in the first sample taken after populating of the roost. In this sample, 77.5% of specimens contained virus, whereas the succeeding 2 samples showed a statistically significant 2- to 8-fold decrease of detection frequency (χ^2 43.4, p<0.001). A second and more significant amplification occurred ≈1 month after parturition, with 100% of collected fecal samples testing positive for CoV RNA (sampling dates 4 and 5). The second peak was characterized by an increase in median RNA concentration by ≈2 orders of magnitude (Table 2). Peak concentration was 2,453,390,770 CoV RNA copies/g of feces. The increased virus detection rate in the post-parturition period in comparison to the preceding 2 sampling dates, as well as the observed increase in virus concentration were statistically highly significant (analysis of variance [ANOVA], F = 24.7, p<0.001; χ^2 107.9, p<0.001).

For AstV, no amplification was associated with parturition in the same samples. Total detection rate of astroviruses was 51.2% before birth of the first pup and 40.5% thereafter. However, prevalence and virus concentration significantly increased in the second sampling than in the first and fourth samplings, respectively (χ^2 7.4, p = 0.006); ANOVA, F = 4.4, p = 0.03). This pattern resembled the amplification after formation of the colony as also observed in CoV. Figure 3, panel B, shows AstV RNA concentrations over time.

Concentration and detection rates of AdV were determined next. As shown in Figure 3, panel C, no marked variation in prevalence was seen. Detection rate was 46.4% before birth of the first pup and 57.7% thereafter. Although statistically significant variation in virus concentrations could be observed (ANOVA, F = 8.2, p<0.001), this was exclusively contributed by slightly lower virus concentrations in the first sampling than in the succeeding samples (Table 2).

Because of the diverging pattern of amplification of the RNA viruses (CoVs, AstVs) against the DNA virus (AdV), the investigation was repeated the next year (2009). All viruses were detected again (Figure 3). Unfortunately, the colony was found to be abandoned after the first postparturition sampling, leaving an incomplete dataset for that year. Still, it could be seen and statistically confirmed that the CoV was beginning to be amplified after parturition (χ^2 7.85, p = 0.005), while no significant variation in prevalence or virus concentration was visible for the other viruses (data not shown).

A repetition of the full sampling scheme was attempted again in 2010. All 5 sampling dates could be completed, yielding a sample of 187 pools in total, equivalent to 935 individual fecal pellets. As shown in Figure 3, the CoV showed the same 2 amplification peaks as observed in 2008, one after formation of the colony and one after parturition. Mean virus concentrations these samples were significantly increased compared with the samples taken at other times (ANOVA, F = 22.0, p<0.001). The detection rate during the first peak was 100.0%, followed by 2-fold and 5-fold decreases 3 and 6 weeks later (χ^2 52.0, p<0.001), and an augmentation to 97.5% after parturition (χ^2 77.7, p<0.001). The maximal CoV concentration in 2010 was higher than in 2008, at 50,495,886,830 RNA copies/g of feces. The amplification pattern of AstV showed clearer similarities to that of CoV in 2010. An initial peak of detection rate was 97.5%, followed by a detection rate of 22.2%–22.4% in

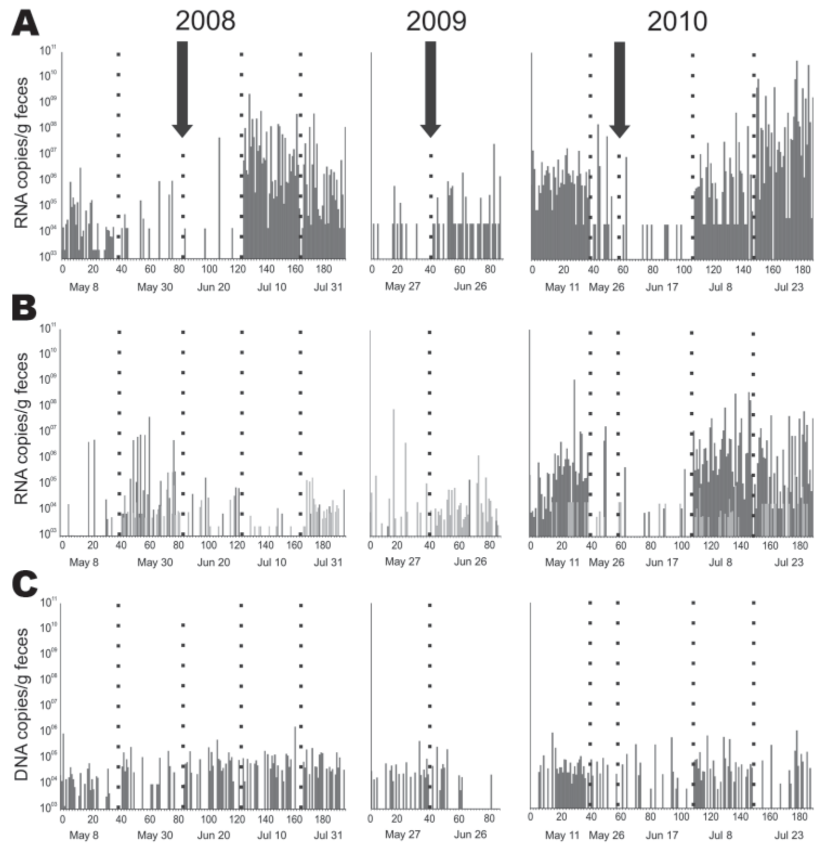

Figure 3. Detection frequency of bat viruses and virus nucleic acid concentrations over time. A) Coronavirus; B) astrovirus; C) adenovirus. Samples were obtained approximately every 3 weeks from the same *Myotis myotis* bat maternity roost in 3 different sampling years, 2008–2010. Each sample was tested by specific real-time reverse transcription–PCR (RT-PCR) with RNA/DNA concentrations per gram of feces given on the y axis. The arrows indicate the time of birth of the first pup. Numbers on the x-axis represent individual fecal pools tested, consisting of 5 single fecal pellets each. Five different sampling dates (below each panel) are shown by dotted lines for each sampling year. Empty columns indicate pools that tested negative. In panel B, light and dark gray bars identify results by 2 different real-time RT-PCRs that were used simultaneously to cover the large astrovirus diversity encountered.

subsequent samples and 97.5%–100% after parturition (χ^2 56.2 and 92.2, respectively, p<0.001). Virus concentrations were significantly increased in these amplification peaks (ANOVA, F = 7.8, p<0.001). The amplification was almost completely contributed by one of the AstV lineages (represented by BtAstV/N58–49), while the other lineages were constant (Figure 3, panel B). Notably, the BtAstV/N58–49 lineage had been present only sporadically in the years before (Figure 3, panel B). Detection frequency for AdV was 58.6% before parturition and 40.3% thereafter without any significant variation in virus concentrations between sampling dates (ANOVA, F = 0.5, p = 0.72).

Effect of Virus Abundance of Bat Reproductive Success

CoV, AstV, and AdV are clearly pathogenic for other mammals. To determine whether the presence of these viruses had any influence on bats' health, the reproductive success of the maternity colony was evaluated in 2008 and 2010. The data are summarized in Figure 4. In a census taken 2008 before parturition, the colony comprised 581 female adult bats. A second census after parturition yielded 394 adults and 220 newborns. The decline in adult females and the moderate number of pups contrasted with observations made in 2010, when 480 adult females were counted before parturition and 437 thereafter, along with 285 pups. The gain in total colony size was significantly greater in 2010 than in 2008 (χ^2 18.3, p<0.001).

Discussion

Viral host switching is probably determined by the chances of interspecies contact, as well as by the concentration and prevalence of virus in the donor species. To judge zoonotic risks associated with bats, when and where these 2 variables would favor transmission must be determined. In this study, we found that strong and specific amplification of the RNA viruses, but not of the DNA virus, occurred upon colony formation and following parturition.

The viruses monitored in our study were selected because they are regularly encountered in bats and thus provide a certain chance of detection. Attempts to characterize virus dynamics in bat populations have been made earlier by using the examples of lyssaviruses (rabies virus and related species), filoviruses (Ebola and Marburg viruses) and henipaviruses (Hendra and Nipah viruses). However, because these viruses are found rarely, only vague conclusions have so far been made. For instance, increased contact between bats and humans through bat migratory events or fruit harvesting periods have been temporally linked with individual human cases of Ebola and Nipah virus infection (*22,23*). One study has shown

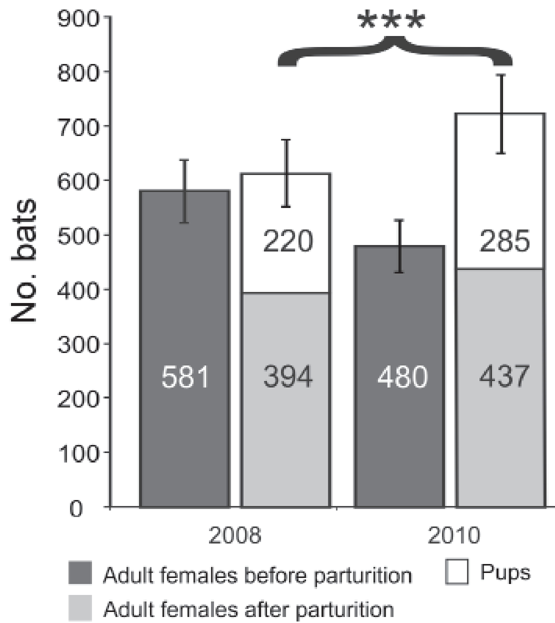

Figure 4. *Myotis myotis* bat maternity roost composition and reproductive success. Age composition of bats composing the *M. myotis* maternity roost under study are depicted before and after parturition in 2 different sampling years, 2008 and 2010. The y-axis represents the number of individual bats, additionally indicated in individual bars. The brace and asterisks represent statistical significance of the gain in total colony size after parturition in 2010, compared with colony size in 2008. Error bars represent an assumed 10% error margin in counting.

that the success of Nipah virus isolation from *Pteropus* spp. bats depended on seasonal factors, which was interpreted as evidence for season-dependent variation of virus concentration or prevalence (*24*). Furthermore, the reproductive cycle of bats has been tentatively connected with seasonality of henipavirus, filovirus and lyssavirus seropositivity in bats as well as with the temporal distribution of Nipah virus outbreaks in humans (*10*,*12*,,*15, 25–27*). Our direct data on virus concentration and prevalence for CoV and AstV integrate many of these independent observations and provide a model that might be transferable to other viruses. The initial peak in annual CoV and AstV prevalence observed in our study was probably due to the formation of a contiguous population of sufficient size and density, bringing together enough susceptible bats to establish a critical basic reproductive rate of infection (*28,29*). The second amplification peak after parturition was most probably associated with the establishment of a susceptible subpopulation of newborn bats who had not yet mounted their own adaptive immunity. Sporadic vertical transmission from mothers to pups as observed in *Pteropus* spp. bats artificially infected with Hendra virus would probably initiate this second wave of infection (*30*). The main driver of the second wave would then be a horizontal transmission between pups. The latency between parturition and the second wave of virus amplification indicates a certain level of perinatal protection conferred by mothers during the first weeks of life as demonstrated for other small mammals, and as indirectly suggested for bats (*13*,*31–33*). This protection may be differentially effective against different viruses, as indicated by the differential amplification patterns between CoVs and AstVs. While CoVs were amplified both in 2008 and 2010, AstVs underwent postparturition amplification only in 2010 when a new virus lineage gained predominance in the population. This finding strongly indicates antigen-specific immune control of virus circulation.

A common, but unproven, assumption is that bats are resistant to even highly pathogenic viruses (*2*,*3*). In this study, we have correlated direct measurements of virus burden with the reproductive success of a bat colony. The rate of successful reproduction is probably a sensitive indicator of the presence or absence of disease, given the tenuous conditions under which bats breed in temperate climates. Indeed, no effects of CoV and AstV on reproduction were initially apparent; although postparturition amplification of both viruses was more efficient in 2010 than in 2008, the overall breeding success was significantly better in 2010. This result may merely have been a consequence of a positive correlation between virus amplification and colony size, which was larger in 2010 due to better breeding success. On the other hand, the individual prenatal amplification peaks of both CoV and AstV were higher in 2010, which may have enabled better perinatal protection and thus better survival of newborns. The grouping of large numbers of pregnant females before birth is a specific characteristic of bats that may contribute to their puzzling ability to maintain highly pathogenic viruses without experiencing die-offs. Our noninvasive approach did not allow any further analyses such as the testing of blood and colostrum samples for antibodies. Nevertheless, the general picture obtained in this study by correlating virus and bat population dynamics suggests that bats control infections in similar ways to other mammals, and that they may well experience virus epidemics.

Another intriguing finding of our study was the difference in the amplification pattern of the RNA viruses and that of the DNA virus. We selected these viruses because, in humans, AdVs are typically capable of persisting in tissue (*34*) and thus do not depend so much on continuous transmission and consistent amplification on the population level. Indeed, it appeared that AdV did not make use of periodic amplification in our bat colony. Persistence on the level of individual bats is more common for DNA viruses than for RNA viruses. RNA viruses ensure that they are maintained on a population level by a

Table 2. Viral RNA detection characteristics over time, Germany, 2008–2010*

Sampling date	No. fecal pellets	Coronavirus			Astrovirus			Adenovirus		
		No. (%) positive	Log abun	Log conc	No. (%) positive	Log abun	Log conc	No. (%) positive	Log abun	Log conc
2008 May 8	40	31 (77.5)	4.13	4.24	11 (27.5)	3.10	3.66	23 (57.5)	3.93	4.17
2008 May 30	44	10 (22.7)	4.01	4.65	32 (72.7)	4.39	4.53	16 (36.4)	4.21	4.65
2008 Jun 20	40	4 (10.0)	3.19	4.19	13 (32.5)	3.92	4.41	22 (55.0)	4.61	4.87
2008 Jul 10	40	40 (100.0)	6.88	6.88	9 (22.5)	2.73	3.37	22 (55.0)	4.59	4.85
2008 Jul 31	31	31 (100.0)	5.76	5.76	22 (71.0)	3.93	4.08	20 (64.5)	4.45	4.64
2009 May 27	40	9 (22.5)	3.73	4.38	14 (35.0)	3.72	4.18	19 (47.5)	4.10	4.42
2009 Jun 26	48	35 (72.9)	4.24	4.38	32 (66.7)	3.90	4.07	11 (22.9)	3.93	4.57
2010 May 11	40	40 (100.0)	6.21	6.21	39 (97.5)	5.41	5.42	27 (67.5)	4.42	4.59
2010 May 26	18	9 (50.0)	4.07	4.37	4 (22.2)	5.75	6.41	7 (38.9)	4.29	4.70
2010 Jun 17	49	10 (20.4)	3.66	4.35	11 (22.4)	3.62	4.26	13 (26.5)	3.85	4.42
2010 Jul 8	40	39 (97.5)	5.79	5.80	40 (100.0)	6.31	6.31	27 (67.5)	4.44	4.61
2010 Jul 23	40	39 (97.5)	7.91	7.92	39 (97.5)	5.58	5.59	12 (30.0)	4.32	4.84

*Abun, median virus concentration multiplied by prevalence; conc, median virus concentration, RNA/DNA copies per gram of feces in positive samples.

much higher error rate of the enzymes they use for genome replication and consequent higher levels of antigenic variability, causing waves of epidemic spread as confirmed for bat-borne RNA viruses in this study. This factor can explain why most emerging viruses, including those from bats, are indeed RNA viruses (*2*,*35*).

For CoV, our study indicates clearly that virus amplification takes place in maternity colonies, confirming our earlier statistical implications from studies in a different region and on a different species (*16*). High peak RNA concentrations in the range of 10^9–10^{10} copies/g were observed, which is tremendously higher than CoV concentrations observed in earlier studies outside the parturition period (*19*). Similarly high RNA virus concentrations are observed in human diseases transmitted through the fecal-oral route, e.g., picornaviruses or noroviruses, which suggests that maternity roosts may involve an elevated risk of virus transmission to other hosts. It is interesting to reconsider the potential genesis of the SARS epidemic in this light. Although an origin of SARS-related CoV in bats is confirmed (*9*,*36*), SARS-CoV precursors have existed in carnivores some time before the SARS epidemic and have been transmitted from carnivores to humans again at least one additional time after the end of the epidemic (*37*,*38*). these data provide an intriguing explanation of how the SARS agent may have left its original reservoir (*39*,*40*). The data also indicate a feasible and ecologically sensible means of prevention. Because carnivores are known to enter maternity roosts to feed on dead newborn bats, bat maternity roosts should be left undisturbed by humans and kept inaccessible to domestic cats and dogs.

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Dr Drexler is a physician and clinical virologist affiliated with the University of Bonn. He is currently working on the implementation of methods for affordable viral load monitoring and the characterization of novel human and zoonotic viruses.

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Epidemiology of *Mycobacterium bovis* Disease in Humans, the Netherlands, 1993–2007

Christof J. Majoor, Cecile Magis-Escurra, Jakko van Ingen, Martin J. Boeree, and Dick van Soolingen

In the Netherlands, 1.4% of tuberculosis (TB) cases are caused by *Mycobacterium bovis*. After we admitted 3 patients with *M. bovis* infections to our reference hospital, we conducted a retrospective analysis of all *M. bovis* disease in the Netherlands during 1993–2007. We analyzed data from 231 patients for clinical, demographic, treatment, and outcome characteristics and for risk factors. Most patients were native Dutch (n = 138; 59.7%) or Moroccan (n = 54; 23.4%). Disease was mainly extrapulmonary (n = 136; 58.9%). Although 95 patients had pulmonary disease, person-to-person transmission did not occur, as shown by structural DNA fingerprinting analysis. Lymph node TB was more likely to develop in women (p<0.0001), whereas pulmonary *M. bovis* disease developed more frequently in men (p<0.0001). Diagnosis was accurate but delayed and led to inadequate treatment in 26% of the cases. Proportion of deaths from *M. bovis* disease was higher than that for *M. tuberculosis* disease.

Mycobacterium bovis disease was common in the Western world in the era before pasteurization of milk products. In 1938, the percentage of *M. bovis* disease among all patients with tuberculosis (TB) was 9% in Amsterdam and 11% in the rest of the Netherlands. In 1940, pasteurization became obligatory, and in 1952, the percentage of *M. bovis* disease had dropped to 1.5%–2.0% in Amsterdam (*1*,*2*). From 1945 until the mid-1960s, *M. bovis* infection was gradually eradicated in livestock in the Netherlands. Nevertheless, during 1973–1975, ≈2.5% of the human TB cases in the Netherlands were still caused by *M. bovis* (*3*). Since 1993, only 10 cases of *M. bovis* infection have occurred in livestock in the Netherlands. All were caused by infected livestock that had been imported (D. Bakker, pers. comm.). Because most *M. bovis* infections are contracted through the oral route, extrapulmonary manifestations were primarily observed.

Diagnosing extrapulmonary TB is generally difficult, and as the prevalence of TB has declined, the experience of physicians in diagnosing this specific infectious disease has also decreased, and therefore the time to diagnosis has increased (*4*,*5*). The difficulties in the diagnosis and treatment of *M. bovis* infections in 3 female patients at the University Centre for Chronic Diseases Dekkerswald prompted the present study. To investigate the magnitude of *M. bovis* infection in persons in the Netherlands, we conducted a retrospective study of patients with *M. bovis* infection and describe the epidemiologic, clinical, and bacteriologic findings.

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Materials and Methods

We retrieved data from 2 databases: one at the National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands, which contains the bacteriologic information of all *M. tuberculosis* complex isolates in the Netherlands. The other is the Netherlands Tuberculosis Register (NTR), an anonymous case register held by KNCV Tuberculosis Foundation in The Hague, the Netherlands. This database is based on voluntary registration but contains data from virtually all TB patients in the Netherlands.

The patients in the NTR database have been registered by their physicians. This register holds basic demographic, clinical, and some bacteriologic data. Death is registered as

TB related or not TB related without further explanation. Treatment and treatment outcome are registered without further details. Exact treatment length could be determined because length was registered in 2 different categories. Therefore, we could categorize treatment length in 6 groups, namely ≤3 months, 4–5 months, 6 months, 7–9 months, 10–12 months, and >12 months.

We selected all *M. bovis* cases that occurred during 1993–2007. By using these 2 databases we had information on bacteriologic and clinical factors, demographic data, and risk factors, as well as treatment and outcome. We used the RIVM database for the bacteriologic data and localization of the infections and the NTR database for the demographic and clinical data, including treatment outcome. To distinguish *M. bovis caprae* and *M. bovis bovis* infections, we reviewed available data on IS*6110* restriction fragment length polymorphism, spoligotyping, and pyrazinamide susceptibility for all *M. bovis* isolates analyzed in this study (*6*,*7*). Because IS*6110* typing of all *M. tuberculosis* complex isolates is routinely performed in the Netherlands, we also analyzed information on possible interhuman transmission of *M. bovis*.

Associations between localization of infection in the patient, geographic distribution, ethnicity, etc. were evaluated on basis of information from both databases by using the χ^2 test. Mortality rates were evaluated by using the NTR database and were correlated with the demographic and clinical data.

Case Reports

Patient 1

A 73-year-old woman sought treatment for nonproductive cough and dyspnea. Medical history included severe rheumatoid arthritis, for which she had received treatment with anti–tumor necrosis factor-α (TNF-α) for 1 year. High-resolution computed tomography (CT) imaging of the thorax showed micronodular lesions in both lungs. CT imaging of the brain indicated several cerebral nodular lesions, suspected to be tuberculomas. Culture of bronchoalveolar lavage fluid yielded *M. bovis*. The patient was treated with isoniazid, rifampin, and ethambutol. Moxifloxacin and steroids were added, because of the high penetration level through the blood–brain barrier of moxifloxacin and to compensate for the absence of pyrazinamide in the treatment regimen.

Patient 2

An 84-year-old woman with a medical history of myelodysplastic syndrome and diabetes mellitus type 2 sought treatment for a traumatic leg wound that did not heal, and her condition gradually progressed to generalized skin lesions. She was referred to the dermatologist in our hospital. Culture of a skin biopsy specimen grew *M. bovis*. The patient had lesions on her tongue, typical of TB (Figure 1), and on her the larynx and in her lungs. The patient's condition resolved after 9 months of treatment with isoniazid, rifampin, and ethambutol. Contact tracing showed no human transmission.

Patient 3

An 87-year-old woman with severe rheumatoid arthritis sought treatment for weight loss, cough, night sweats, and dyspnea 1 year after starting anti–TNF-α treatment. Radiologic imaging showed a single nodular lesion of the lung parenchyma and pleural fluid. Culture of a bronchoalveolar lavage specimen yielded *M. bovis*. The patient received isoniazid, ethambutol, and moxifloxacin for 18 months because she had an intolerance for rifampin. No contact tracing was performed**.**

Results

During 1993–2007, a total of 16,059 patients were registered in the NTR with culture-proven TB; 231 (1.4%) patients were registered with *M. bovis* infection (*8*) (Figure 2). The number of patients with *M. bovis* infection born outside the Netherlands (foreign-born) showed an increase in the proportion from 35.1% to 46.3% in the 1ast 5 years of the study period (Figure 3). Over time, the percentage of patients with *M. bovis* TB who live in major cities increased from 33.3% to 41.8%, with an accompanying decline elsewhere in the Netherlands. No relation was found between outbreaks in livestock and human disease.

Among patients with *M. bovis* disease, a significant difference in mean age was noted among those born in the

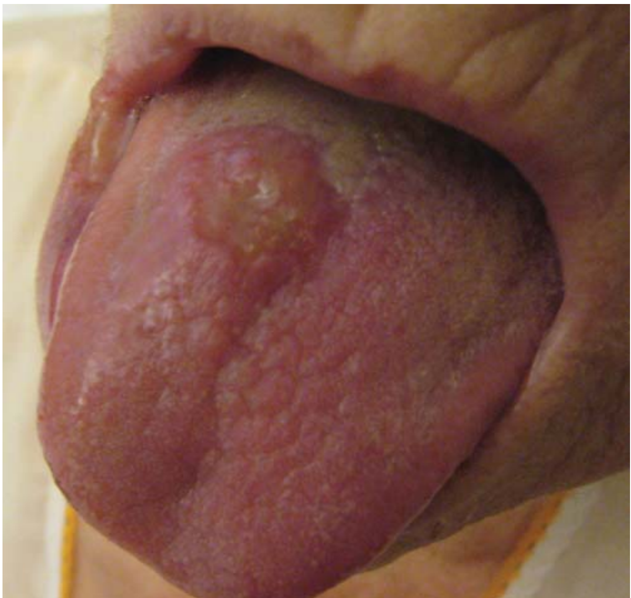

Figure 1. *Mycobacterium bovis* lesion on the tongue of patient 2, the Netherlands.

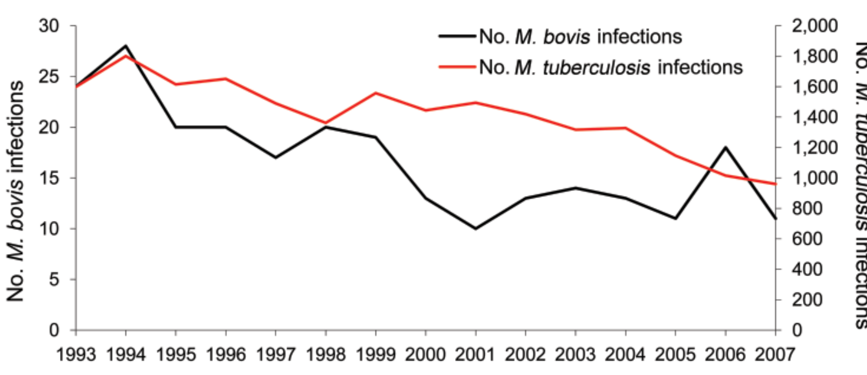

Figure 2. *Mycobacterium tuberculosis* and *M. bovis* infections, the Netherlands, 1993–2007. Data derived from the National Institute for Public Health and the Environment (RIVM) database.

Netherlands and immigrants. In native Dutch persons with *M. bovis* disease, the median age was 64.8 years, whereas among immigrants, the median age was 38.6 years (Figure 4). We observed 58 cases in young foreign-born patients and 16 cases in young Dutch patients (<40 years). Five Dutch patients had a general risk factor for contracting *M. tuberculosis* complex disease (1 with drug addiction, 1 with alcohol addiction, and 3 with risk factors not specified) and 44 foreign-born had 1 or 2 general risk factors (32 immigrants, 8 with fugitive status, 3 illegal immigrants, and 1 TB contact, 1 in criminal detention, 1 with risk factor not specified).

Most of the patients were native Dutch (138 [59.7%] of 231). Most foreign-born patients came from Morocco (54 [23.4%] of 231). The other immigrants had other places of origin: Europe (n = 6; 2.6%), Africa (n = 15; 6.5%), Asia (n = 9; 3.9%), and South America (n = 3; 1.3%) (Table 1). A distinction between native Dutch and second-generation Dutch immigrants could not be made.

M. bovis TB mainly had appeared as extrapulmonary disease; 136 (58.9%) of 231 patients had extrapulmonary disease alone, 68 (29.4%) of 231 had pulmonary *M. bovis* disease, and 27 (11.7%) of 231 had both pulmonary and extrapulmonary disease. A significant difference between men and women was found in terms of localization of disease. In women, lymph node TB was more likely to develop, mainly in the cervical region (p<0.0001); men more often had pulmonary *M. bovis* disease (p<0.0001). This finding was seen in all age groups in both Dutch and foreign-born patients. (Table 1)

Several treatment schedules were used for our study population; 157 of 231 patients received a combination of isoniazid, rifampin, ethambutol, and pyrazinamide, or another pyrazinamide-containing antimicrobial drug combination. Treatment with pyrazinamide-containing regimens had no negative effect on treatment outcome.

According to World Health Organization (WHO) standards, a regimen of isoniazid and rifampin for 9 months and ethambutol for 2 months is indicated for treating *M. bovis* disease. In our study population, we noted that 113 (52%) of 217 patients were cured and completed treatment according to the WHO standard. Thirty-seven (17%) of 217 patients were considered cured with an insufficient treatment schedule, duration, or both. For 14 patients, outcome data were not available. No significant associations were found between insufficient treatment and particular disease localizations or outcome.

An anonymous questionnaire distributed to the physicians who were caring for the patients at the time of disease (34 sent and 24 returned) showed that most of the physicians followed the Dutch TB guidelines, which are based on the WHO standard. A problem these colleagues

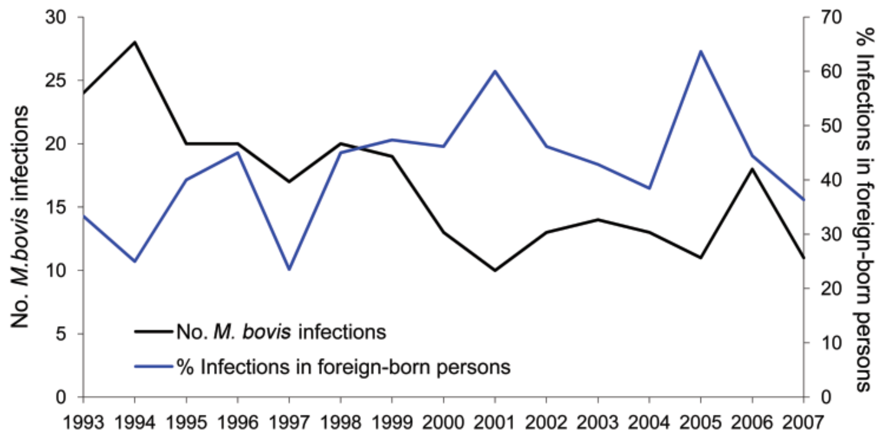

Figure 3. Percentage of *Mycobacterium bovis* infections in foreign-born persons and total number of *M. bovis* infections, the Netherlands, 1993–2007. Data derived from the National Institute for Public Health and the Environment (RIVM) database.

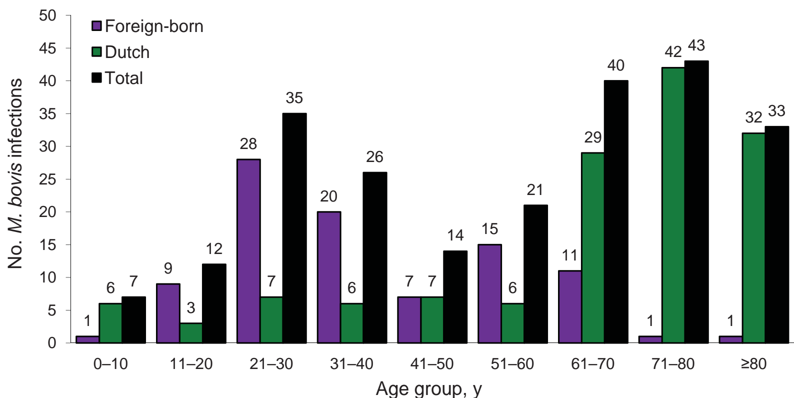

Figure 4. Number of *Mycobacterium bovis* infections, according to patient age and origin, the Netherlands, 1993–2007. Data derived from the National Tuberculosis Registry database.

faced was the time lag between sampling and the results of culture, identification, and drug susceptibility tests, which sometimes took as long as 4 months.

Treatment outcome comparisons for Dutch and foreign-born patients in the Netherlands are shown in Table 2. The overall proportion of deaths for the infection itself was 5.2% for *M. bovis* disease, and the proportion of deaths associated with non–TB-related causes (e.g., cardiac disease and hematologic malignancy) for patients with *M. bovis* disease was 14.7%.

An analysis of the overall deaths from bovine TB according to sex, age, ethnicity, and disease localization is shown in Table 3. A correlation was found between death and age >60 years, Dutch nationality, and miliary disease. When dividing deaths into the categories of TB related and not–TB related, only high age was statistically significant between the groups; all other variables were not significant (Table 4). Cause and rate of death differed between sexes: among women, death was more often related to *M. bovis* TB; in men, the overall mortality rate was higher, although these differences were not statistically significant.

Discussion

M. bovis TB comprised 1.4% of all TB cases in the Netherlands during 1993–2007. This finding is comparable to those of studies in other countries where control and corresponding control efforts of *M. bovis* TB in livestock are present (*9–11*). During 1993–1997, mainly elderly Dutch persons were found to have bovine TB; later (1998–2007), the infections were divided more equally between the native Dutch and immigrants (Figure 3).

M. bovis TB in the population of the Netherlands shows an age curve with double peaks. The younger patients were mostly foreign-born or first- and second-generation immigrants, who may have (frequently) traveled back to their country of origin or contracted an *M. bovis* infection by the oral route before coming to the Netherlands. These patients more often had extrapulmonary *M. bovis* disease, which mainly affected the cervical or abdominal lymph nodes. Ingestion of unpasteurized dairy products is the most likely route of infection (*12*,*13*). The 12 Dutch patients, ages 20–40 years, may have been in contact with nonpasteurized food while traveling to developing countries because the chance of contracting a *M. bovis* infection in the Netherlands is considered low.

The second age peak contains elderly native-born Dutch persons. This result is most likely a birth-cohort effect because these persons most probably had a late endogenous reactivation of latent *M. bovis* infection contracted in the era before pasteurization of milk was introduced and while *M. bovis* infection in livestock was still highly prevalent in the Netherlands. Spoligotyping of their isolates showed a single typical pattern, which is considered the old predominant cattle-endemic strain of the Netherlands (*14*). This epidemiologic trend has also been seen in other European countries, including Norway, Sweden, and Belgium (*9*,*10*).

Endogenous reactivation of *M. bovis* infections in elderly patients follows impairment of immunity from hematologic causes, immune modulation by medication (including anti–TNF-α treatment), other coexisting conditions, or immunosenescence. As the use of anti–TNF-α treatment rises, due to increasing indications of rheumatologic and gastrointestinal diseases, mycobacterial disease will likely become more common (*15*). Two of the patients we described had negative tuberculin skin test (for *M. tuberculosis* complex) and chest radiograph results and, therefore did not receive isoniazid prophylaxis before they received anti–TNF-α treatment. This result calls into question the efficacy of the screening protocol used in the Netherlands to evaluate patients before they receive anti–TNF-α treatment. Of note, reactivations of latent TB usually occur in the first 3–4 months of anti–TNF-α therapy (*15*), although in our 2 *M. bovis* patients, reactivation occurred after 1 year. This long-term asymptomatic reactivation of disease has been previously observed (*16*,*17*) and raises questions involving the role of the bacteriologic virulence factor in the pace of the reactivations.

Table 1. Clinical and demographic data for *Mycobacterium bovis* TB case-patients, by sex, as recorded in the Netherlands Tuberculosis Registry, the Netherlands, 1993–2007*

Characteristic	Dutch, no. (%), n = 138	Foreign-born, no. (%),† n = 93	Total, no. (%), N = 231
All case-patients			
M	56 (40.6)	49 (52.7)	105 (45.5)
F	82 (59.4)	44 (47.3)	126 (54.5)
Age, y			
0–60			115 (49.7)
M	13	45	
F	22	35	
>60			116 (50.3)
M	43	4	
F	60	9	
Localization			
Pulmonary			80 (34.6)
M	26	25	
F	22‡	7§	
Respiratory tract			19 (8.2)
M	5	5	
F	8	1	
Meningeal and CNS			6 (2.5)
M	2	0	
F	F	0	
Intestinal tract			13 (5.6)
M	0	3	
F	5	5	
Bone and joint			12 (5.2)
M	4	2	
F	5	1	
Urogenital tract			13 (5.6)
M	4	3	
F	4	2	
Other organs (e.g., lymph nodes)			59 (25.5)
M	5	9	
F	24¶	21#	
Miliary TB			11 (4.8)
M	6	0	
F	3	2	
Unknown			18 (7.8)
M	4	2	
F	7	5	

*TB, tuberculosis; CNS, central nervous system.
†Foreign-born patients originated from other European countries (n = 6), Africa (n = 69), Asia (n = 9), South America (n = 3), and unknown areas (n = 6).
‡p = 0.018.
§p<0.001.
¶p = 0.004.
#p = 0.002.

In the Netherlands, the 2 largest immigrant populations are from Turkey and Morocco (378,330 and 341,528 persons, respectively) (*18*). A high occurrence of *M. bovis* disease was observed in Moroccan patients, whereas, to our surprise, *M. bovis* disease was rare in Turkish patients. Both populations come from agricultural areas where pasteurization of dairy products is not common. Popular raw milk cheeses in Morocco (*jben*) and Turkey (*kasar, tulum*) have been found to contain *Listeria* and *Brucella* species in as many as 8.2% of the samples tested (*19*,*20*). In both studies, cheeses were not tested for *M. bovis*. However, *M. bovis* can survive in raw milk cheese and cause an infection after it is eaten, as was described recently for a cluster of infections resulting from consumption of fresh Mexican cheese in New York and San Diego (*12*,*13*,*21*). Besides consumption of fresh cheese and unpasteurized milk, consumption of raw or undercooked meat is also a possible route of oral transmission (*10*,*22*,*23*).

More women than men were infected by *M. bovis*, contrary to the epidemiology of *M. tuberculosis*. Other studies about *M. bovis* do not show this result (*9*,*11*). This difference could be an age-related effect for Dutch patients; however, one can also see this difference in the Moroccan patient population (data not shown).

Notably, we also found a difference in disease localization between men and women. More cervical lymph node TB, which was not correlated with age, was diagnosed for female patients. Female sex has in general been considered a risk factor for *M. tuberculosis* extrapulmonary TB (*24*–*26*). However, this finding has not been described for *M. bovis* TB. Various explanations have been given for this gender difference in TB. One possible explanation could be a difference in the immunity to *M. tuberculosis* complex infection. Several studies have been conducted in humans to compare the immune response in both sexes. Differences in reaction among others in TNF and interleukin-10 production have been found (*27*,*28*). Another explanation for this finding may be related to the route of infection. Men could be more likely to become infected through the tracheal route by aerosols from diseased cattle, whereas women become infected by ingesting *M. bovis* while preparing or consuming contaminated food. Lastly, smoking has also been identified as a risk factor for pulmonary TB (*25*), which (assuming a higher percentage of men are smokers) could explain the difference we found in our study that men have more pulmonary *M. bovis* disease than women. Unfortunately, we could not retrieve smoking status from the databases.

The geographic distribution of *M. bovis* TB in the Netherlands showed a proportional increase in *M. bovis* infections in the major cities in the western part of the Netherlands during 1993–2007. This finding probably reflects the population demographics of the Netherlands, where most persons live in and between major cities, but also by the facts that immigrants mainly live in major cities and that the elderly persons who contracted *M. bovis* TB in the prepasteurization era are undergoing a natural decline in health.

We showed that, in 37 (17.1%) of the 217 patients, treatment of *M. bovis* disease cases in this study was not compliant with international guidelines. Although all *M.*

Table 2. Treatment results according to age, sex, and localization of *Mycobacterium bovis* disease in Dutch and foreign-born case-patients, as recorded in the National Tuberculosis Registry, the Netherlands, 1993–2007*

Variable	No. (%) Dutch patients, n = 138				No. (%) foreign-born patients, n = 93			
	Treatment completed	Cause of death		Other†	Treatment completed	Cause of death		Other†
		TB-related	Non–TB-related			TB-related	Non–TB-related	
Total	85 (61.6)	10 (7.2)	28 (20.3)	15 (10.9)	66 (71.0)	2 (2.1)	6 (6.5)	19 (20.4)
Age, y								
0–60	25	1	3	6	59	2	3	16
>60	60	9	25	9	7	0	3	3
Sex								
M	33	3	18	2	35	0	3	11
F	52	7	10	13	31	2	3	8
Localization								
Pulmonary	26	2	9	3	21	0	2	5
Extrapulmonary	52	5	11	10	44	0	3	11
Pulmonary and extrapulmonary	7	3	8	2	1	2	1	3

*TB, tuberculosis.
†Treatment not completed or treatment continued elsewhere.

Table 3. Correlation of overall deaths from *Mycobacterium bovis* disease with demographic variables, the Netherlands, 1993–2007*

Variable	p value
Sex	0.31
Age >60 y	<0.0001
Dutch nationality	0.001
Disease localization (miliary TB)	<0.0001

*χ^2 test. TB, tuberculosis.

tuberculosis complex isolates in the Netherlands undergo drug susceptibility testing, apparently the results of such testing do not reach clinicians. Recognition of *M. bovis* as the causative agent of TB does not in itself lead to the conclusion that adjustment of the therapy is required; many physicians only make adjustments after receiving the results of drug susceptibility testing, which usually arrive quite late because of the slow growth of *M. bovis*. Moreover, the quick molecular identification of cultured *M. bovis* still implies a culture delay of ≈4 weeks. Better education of physicians and increasing future application of direct molecular identification of *M. bovis* from clinical samples, i.e., sputum or lymph node aspirates**,** is therefore needed.

The overall proportion of deaths among patients with *M. bovis* disease was higher (19.9%) than that among patients with *M. tuberculosis* disease (4.4%) in the Netherlands. The mortality rates during 1993–2007 were 5.2% for *M. bovis* disease versus 1.9% for *M. tuberculosis* disease. This result is probably related to the higher prevalence of miliary and central nervous system localization of *M. bovis* disease. Death rates from other, non–TB-related, causes for patients with *M. bovis* infection were 14.7% compared with 2.5% for *M. tuberculosis* infection (*8*). Subgroup analysis of *M. bovis* showed that the proportion of deaths was higher among Dutch than among foreign-born patients, but this result most likely correlates with age and impairment of immunity (*10*,*11*,*29*).

We cannot explain the unexpected trend in lower survival of female patients after an episode of *M. bovis* disease. Previous studies have shown that male sex is a risk factor for unsuccessful treatment (*30*,*31*), but the numbers in our study were too small to draw any firm conclusions.

This study had limitations. First, the analysis of the results was hampered by the relatively low number of foreign-born patients (n = 93) and the heterogeneous nature of the study population. Therefore, only data concerning patients from Morocco (n = 54) could be analyzed separately, and differences with the Dutch case-patients were found in terms of age, mortality rate, and primary localization of disease.

Another limitation is that a distinction could not be made between native Dutch and second-generation immigrants born in the Netherlands. This lack of distinction implies that some patients have roots in a foreign country where the risk for *M. bovis* TB could be higher.

In conclusion, the incidence of *M. bovis* disease in the Netherlands is comparable to that in other countries in which control programs for *M. bovis* infection are enforced. Gender differences in clinical features and mortality rates were found in our cohort of patients. The disease now mainly infects immigrants from Morocco and elderly Dutch citizens. Anti-TNF-α treatment is an emerging cause of endogenous reactivation of *M. bovis* disease in elderly Dutch patients, as occurred in 2 of the recent bovine TB

Table 4. Correlation of TB-related and non–TB-related deaths according to demographic variables, the Netherlands, 1993–2007*

Variable	p value
Sex	0.58
Age >60 y	0.03
Dutch nationality	0.91
Disease localization (miliary TB)	0.49

*χ^2 test. TB, tuberculosis.

cases described in this article; reactivation may be slower than for *M. tuberculosis* infection.

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Molecular Epidemiology of *Fonsecaea* Species

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To assess population diversities among 81 strains of fungi in the genus *Fonsecaea* that had been identified down to species level, we applied amplified fragment-length polymorphism (AFLP) technology and sequenced the internal transcribed spacer regions and the partial cell division cycle, β-tubulin, and actin genes. Many species of the genus *Fonsecaea* cause human chromoblastomycosis. Strains originated from a global sampling of clinical and environmental sources in the Western Hemisphere, Asia, Africa, and Europe. According to AFLP fingerprinting, *Fonsecaea* isolates clustered in 5 groups corresponding with *F. pedrosoi*, *F. monophora*, and *F. nubica*: the latter 2 species each comprised 2 groups, and *F. pedrosoi* appeared to be of monophyletic origin. *F. pedrosoi* was found nearly exclusively in Central and South America. *F. monophora* and *F. nubica* were distributed worldwide, but both showed substantial geographic structuring. Clinical cases outside areas where *Fonsecaea* is endemic were probably distributed by human migration.

The genus *Fonsecaea* comprises etiologic fungal agents of human chromoblastomycosis (*1–3*), a chronic cutaneous and subcutaneous infection characterized by slowly expanding nodules that eventually lead to emerging, cauliflower-like, mutilating and disfiguring eruptions. Infection proceeds with muriform cells in tissue provoking a granulomatous immune response. In areas where it is endemic, disease incidence is high. Yegres et al. (*4*) and Yëgues-Rodriguez et al. (*5*) noted a frequency of 16 cases/1,000 populaAtion under arid climatic conditions in rural communities of Venezuela; chromoblastomycosis in that region is caused mainly by *Cladophialophora carrionii*. In contrast, *Fonsecaea* spp. are prevalent in humid tropical climates. Esterre et al. (*6*) reported 1,343 cases of chromoblastomycosis from Madagascar, 61.8% of which were caused by *Fonsecaea* spp. Kombila et al. (*7*) reported 64 cases in Gabon (equatorial Africa), all caused by *Fonsecaea* spp., and Silva et al. (*8*) cited 325 cases in the Amazon region of Brazil, 98% of which had *Fonsecaea* spp. as the etiologic agent. In Sri Lanka, 94% of 71 chromoblastomycosis cases were caused by *Fonsecaea* spp. (*9*).

Fonsecaea contains anamorphic ascomycetes belonging to the family *Herpotrichiellaceae* (order *Chaetothyriales*), which includes black yeasts and relatives (*10–12*). The genus comprises 3 sibling species: *F. pedrosoi*, *F. monophora*, and *F. nubica,* each of which has pathogenic potential (*10*,*13*,*14*). Infection process and routes of dispersal are insufficiently clarified. Humans presumably acquire the infection after being pricked by contaminated thorns or wood splinters, but some agents are substantially more clinically prevalent than their predominantly (hitherto unnamed) environmental counterparts (*15*), which indicates that infection is not a random process. In many published case reports, etiologic agents were referred to as *Phialophora pedrosoi* or identified with the obsolete name *F. compacta*, now known to be a mutant *F. pedrosoi* (*9*,*13*,*16*). Strains are no longer accessible for molecular verification. Hence, no data are available on the epidemiology of the species as defined by sequence data.

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Phylogenetically, *Fonsecaea* spp. agents of chromoblastomycosis are flanked by nonpathogenic species (*10*) growing on plant debris. Discovery of natural habitat and source of infection by entities emerging on the human host is essential for understanding the evolution of pathogenicity. We present an amplified fragment-length polymorphism (AFLP) DNA fingerprinting study of a worldwide collection of clinical isolates that were identified as *Fonsecaea* spp. by state-of-the-art sequencing methods, supplemented with environmental isolates of the same species. The AFLP technique is a powerful method for discrimination between fungal species and for providing high-resolution fingerprinting data within species (*17–19*).

Materials and Methods

Fungal Strains and Culture Conditions

We studied 81 isolates representing the 3 currently recognized *Fonsecaea* spp. Geographic origins and hosts of the strains are listed in Table 1; the set include reference strains from the Centraalbureau voor Schimmelcultures (CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands) and fresh isolates from patients and from the environment. Stock cultures were maintained on slants of 2% malt extract agar and oatmeal agar at 24°C.

DNA Extraction and Identification

Approximately 1 cm^2 of 14- to 21-day-old cultures were transferred to 2 mL Eppendorf tubes containing 400 µL TEx buffer (Sigma-Aldrich, Zwijndrecht, the Netherlands), pH 9.0 (100 mmol Tris, 40 mmol Na-EDTA) and glass beads (Sigma G9143, Sigma-Aldrich). The fungal material was homogenized with a MoBio vortex (Bohemia, New York, USA) for 1 min. Subsequently, 120 µL of a 10% sodium dodecyl sulfate solution and 10 µL proteinase K (10 mg/mL, Sigma-Aldrich) were added and incubated for 30 min at 55°C; the mixture was vortexed for 3 min. After addition of 120 µL of 5M NaCl and 1/10 vol 10% cetyltrimethylammonium bromide solution (Sigma-Aldrich), the material was incubated for 60 min at 55°C. Then the mixture was vortexed for 3 min. Subsequently, 700 µL SEVAG (24:1, chloroform: isoamyl alcohol) was added, mixed carefully, and centrifuged for 5 min at 4°C at 20,400 × *g*. The supernatant was transferred to a new Eppendorf tube with 225 µL 5M NH_4 acetate (Sigma-Aldrich), mixed carefully by inverting, incubated for 30 min on ice water, and centrifuged again for 5 min at 4°C at 20,400 × *g*. The supernatant was then transferred to another Eppendorf tube with 0.55 vol isopropanol and centrifuged for 5 min at 20,400 × *g*. Finally, the pellet was washed with 1 mL ice cold 70% ethanol. After drying at room temperature, it was resuspended in 48.5 µL TE buffer (Sigma-Aldrich) (Tris 0.12% wt/vol, Na-EDTA 0.04% wt/vol) and 1.5 µL of RNase (Sigma-Aldrich) and incubated in 37°C for 20–30 min. Quality of genomic DNA was verified on agarose gel. Species were identified on the basis of internal transcribed spacer (ITS), partial cell division cycle (*CDC42*), β-tubulin (*BT2*), and ACT sequences (*10–14*).

AFLP Fingerprinting

We followed a protocol provided by the manufacturer (Applied Biosystems, Nieuwerkerk aan de IJssel, the Netherlands), with some minor modifications (*20–23*). Analyses were performed with 100–200 ng DNA.

Restriction and Ligation of Adaptors

Two µL of DNA (100 ng/µL) was added to 9 µL restriction and ligation mixture (1.1 µL T4 DNA ligase buffer [Applied Biosystems]), 1.1 µL M NaCl, 2 U *Mse*I endonuclease, 10 U *Eco*RI endonuclease (New England Biolabs, Ipswich, UK), 30 U T4 DNA ligase, 1 µL *Mse*I-adaptor, 1 µL *Eco*RI-adaptor, and 3 µL $dH_2 0$ and incubated at 37°C for 2.5 h. Subsequently, each restriction/ligation reaction was diluted ≈3× by adding 25 µL demineralized water.

Preselective and Selective PCR

In preselective PCR, 2 µL of diluted restriction/ligation product was added to 7.5 µL of AFLP core mix (Applied Biosystems), 0.25 µL of the *Eco*RI core sequence (5′-GAC TGC GTA CCA ATTC-3′), and 0.25 µL of the *Mse*I core sequence (5′-GAT GAG TCC TGA GTAA-3′). The mixture was amplified in an iCycler (Bio-Rad, Hercules, CA, USA) under the following conditions: 2 min at 72°C, followed by 20 cycles of 20 s at 94°C, 30 s at 56°C, and 2 min at 72°C. Each preselective PCR was diluted 2× by adding 10 µL of dH_2O. In selective PCR, 1.5 µL of diluted preselective PCR products was mixed with 8.5 selective PCR mix containing 0.5 µL *Eco*RI-AC (labeled with FAM [6-carboxy fluorescein]), 0.5 µL *Mse*I-A, and 7.5 µL AFLP core mix (Applied Biosystems). The selective PCR conditions were cycling for 2 min at 94°C, followed by 10 cycles of 20 s at 94°C and 30 s at 66°C (decreasing 1°C with each subsequent cycle), and a final extension of 2 min at 72°C. This sequence was followed by 25 cycles of 20 s at 94°C, 30 s at 56°C, and 2 min at 72°C, and a final incubation of 30 min at 60°C.

AFLP Analysis

FAM-labeled products were prepared for analysis in an ABI PRISM 377 Genetic Analyzer (Applied Biosystems) as follows: the selective PCR products were cleaned with Sephadex G-50, and selective PCR products were mixed with LIZ 500 in the new plate by several times pipetting (first by preparing master mix [8.7 µL demineralized water plus 0.3 µL Liz 500], then mixing this with 1.0 µL

of selective PCR product by pipetting). The total volume was adjusted to 10 μL with dH_2O. Denaturation was done at 95°C for 5 min, and then the reaction was snap-cooled on ice water. The LIZ 500 internal size standard in each sample was used for normalization of the fingerprint pattern according to the instruction manual. The densitometric curves were analyzed with BioNumerics software package (version 4.61, Applied Maths, Kortrijk, Belgium), by using the cosine similarity coefficient and the unweighted pair group method with arithmetic means cluster analysis. Statistical reliability of the cluster was investigated by using a cophenetic value, which calculates the correlation between the calculated and the dendrogram-derived similarity. Subdivisions in clusters were checked visually if they were supported by the banding patterns.

Results

Profiles of 81 strains were generated with the *Eco*RI-AC + *Mse*I-A PCR adaptors. Fingerprints contained ≈60–70 bands in a 50–500-bp range. Another selective PCR with *Eco*RI core sequence+C and *Mse*I core sequence+A primer combination used elsewhere in related fungi (*24*) resulted in nonscorable fingerprints because of amplification of too many or only faint bands. Dendrograms derived from the AFLP banding patterns of *Fonsecaea* spp. were generated by using the unweighted pair group method with arithmetic means cluster analysis (online Appendix Figure, www.cdc.gov/EID/17/3/464-appF.htm). At $\geq$62.50% similarity, 3 main clusters were found that matched with existing species on the basis of multilocus sequence analysis (ITS, *CDC42*, *BT2,* and *ACT1*), i.e., *F. pedrosoi*, *F. monophora*, and *F. nubica*. At an automatic cutoff value option set at <62.5% similarity, the *F. monophora* and *F. nubica* clusters were subdivided in 2 evident groups each, leading to a total of 5 clusters (1–5) interpreted as populations. Clusters 1 and 2 matched with *F. nubica*, clusters 3 and 4 with *F. monophora*, and cluster 5 with *F. pedrosoi*. Individual bands varied within the profiles, but further subclustering was limited, e.g., in a slightly deviating derived subclade in population 5. The groups defined above by AFLP analysis are interpreted as populations (1–5) in the text below. In population 5, some strains were nearly 100% identical, e.g., CBS 122341, 122343, 122345, and 122349, all originating from patients with chromoblastomycosis in Mexico City, Mexico (online Appendix Figure; online Appendix Table, www.cdc.gov/EID/17/3/464-appT.htm).

We determined the geographic distributions of the 5 main populations of *Fonsecaea* strains (Figure). Areas endemic for *Fonsecaea*, judging from the literature, are in tropical and subtropical climate zones. Population 1 comprised a cluster of *F. nubica* strains originating from humans with chromoblastomycosis in Guangdong, People's Republic of China. Population 2 of the same species comprised 4 strains, 2 of which originated from humans with chromoblastomycosis in South America, 1 from France, and 1 with unknown origin. The profiles were too different to trace to any clonal identity. Population 3 (*F. monophora*) comprised 15 strains, most of which were isolated from humans with chromoblastomycosis in South America; 1 originated from the United States, and 1 originated from Haikou in southern China. Two strains were isolated from decaying plants in Brazil, and the second US strain was derived from a human with a brain infection. Two other strains from human brain infections in Brazil and in Africa had unique profiles that could not be unambiguously linked to any other isolate. Another African strain, from a patient with chromoblastomycosis who lived in Spain and had acquired the infection 36 years earlier in Guinea (*25*), also had a unique profile. Population 4 of *F. monophora* comprised 16 strains from Guangdong in southern China, and 1 came from Shandong, ≈1,850 km distant. All had derived from humans with chromoblastomycosis. A single

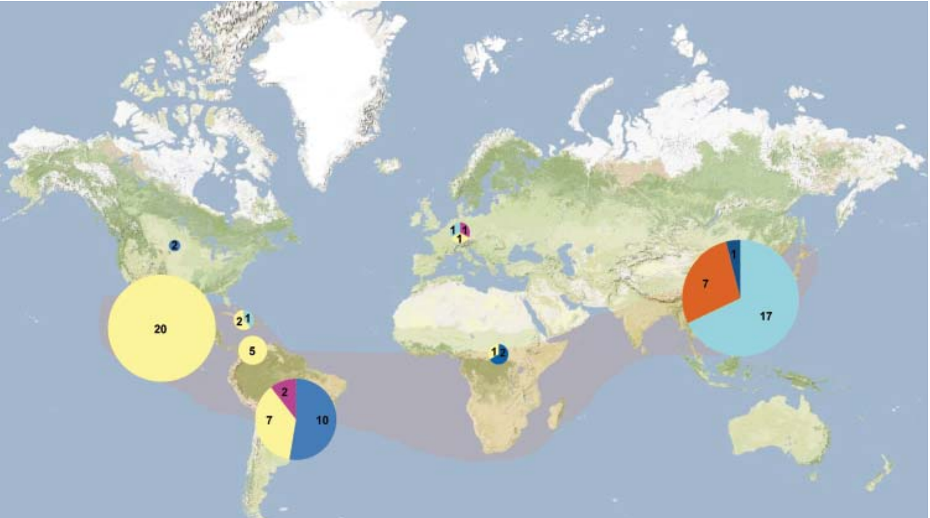

Figure. Geographic distribution of *Fonsecaea* spp. samples analyzed by using amplified fragment-length polymorphism. Light pink shading indicates zone of clinical *Fonsecaea* spp. endemicity, according to published case reports. Sizes of pies and numbers reported within the pies denote the number of strains examined; colors represent *Fonsecaea* spp. populations: orange, *F. nubica* population 1; fuchsia, *F. nubica* population 2; dark blue, *F. monophora* population 3; light blue, *F. monophora* population 4; yellow, *F. pedrosoi* population 5.

sample originated from a patient with a brain infection who lived in the United Kingdom (*26*); whether the patient had visited southern China could not be established. In population 5 (*F. pedrosoi*), most strains originated from chromoblastomycosis patients in Central and South America. Some geographic clustering was visible, i.e., the derived group of strains from South America (uppermost clade of population 5 in the online Appendix Figure) was segregated from those from Central America. Several of the strains from South American originated from soil and were isolated through mouse passage. One strain from an ear of a gazelle in Libya and 1 from a human with chromoblastomycosis in the Netherlands could not directly be linked to any other strain.

Discussion

AFLP typing is comparable to use of other DNA markers, such as random amplified polymorphic DNA, restriction fragment-length polymorphism, or microsatellites, in terms of time and cost efficiency, reproducibility, and resolution (*27*). The technique has emerged as a major epidemiologic tool with broad application in ecology, population genetics, pathotyping, DNA fingerprinting, and quantitative trait loci mapping (*28*). AFLP fingerprinting is useful for the molecular characterization of microorganisms with relatively large genomes, including various fungal species (*18*,*19*,*21–23*,*29*,*30*). In a preliminary experiment that used different primer combinations, the combination *Eco*RI-AC + *Mse*I-A adaptors gave excellent results, yielding readable profiles with well-separated bands.

The degree of variation in *Fonsecaea* appeared to differ between species. The major 5 clusters were separated at <62.5% similarity, with significant differences in the presence of major fragments, several of which were unique to individual isolates or subpopulations. Populations 1 and 2, 3 and 4, and 5 corresponded with species borderlines established recently by Najafzadeh et al. (*10*,*14*) on the basis of multilocus sequencing with ITS, *CDC42*, *BT2*, and *ACT1*. Population 5 (*F. pedrosoi*) varied least at >71.7% similarity, with limited reproducible substructure being discernable. Nearly all isolates of this species originated from South and Central America (Venezuela, Brazil, Mexico, Argentina, Puerto Rico, and Uruguay). One isolate from a human with chromoblastomycosis in the Netherlands was likely to have been imported (*13*). One isolate from a gazelle ear in Libya, northern Africa, was the only geographic exception that could not be explained. Clusters of strains that could be grouped as being visually identical and with similarities >71.7% (online Appendix Figure; online Appendix Table) were mostly collected at close geographic distance from each other. This finding suggests that vectors of dispersal for *Fonsecaea* spp. are slow, leading to detectable regional diversification. The relatively low degree of variation of *F. pedrosoi* and confinement to Central and South America indicate a founder effect, the species being the most recently emerged taxon in *Fonsecaea*. *F. monophora* and *F. nubica* were distributed worldwide but were geographically diverse in that population 4 of *F. monophora* was nearly confined to China, with highly similar profiles (online Appendix Figure). One strain of this population 4, CBS 117238, originated from a brain infection in a human in the United Kingdom; whether this patient had emigrated from China could not be determined from the original publication (*25*). *F. monophora* population 3 was found mainly in the Western Hemisphere, particularly in Brazil. Judging from the near identity of profiles of strains isolated in 1937 (CBS 271.37) and in 1999 (CBS 102245) (online Appendix Figure), we can conclude that clones are maintained locally over decades. The 2 US strains presumably derived from immigrants from South America or Central America. Population 3 was also found in Africa and in Haikou in China, 600 km from Guangdong, where population 4 of *F. monophora* is prevalent. Strains of *F. nubica* show a similar bipartition over Asia and the Western Hemisphere, with a prevalently Chinese (population 1) and a prevalently Brazilian (population 2) population, and a presumed infected immigrant in France. Kawasaki et al. (*31*,*32*) provided similar data on the basis of restriction fragment-length polymorphism of mitochondrial DNA, showing that *Fonsecaea* spp. from Japan and China differed consistently from isolates from Central and South America.

Nearly all *Fonsecaea* spp. isolates available in culture collections originate from mammals, mostly humans with chromoblastomycosis, and were rarely recovered from the environment of symptomatic patients despite several attempts (*33*). Occasionally, *F. pedrosoi* was isolated from mice that were euthanized for isolation of black yeasts after they had been inoculated with environmental samples (*34*). This information suggests that *Fonsecaea* spp., particularly *F. pedrosoi*, have a competitive advantage by using this enrichment source. Mouse passage proved to be more efficient for environmental isolation of etiologic agents of chromoblastomycosis than general methods such as oil flotation (*35*). The latter technique mostly isolates other environmental *Fonsecaea* spp. that are not known to be pathogenic to humans (*33*).

In humans with chromoblastomycosis, the male:female ratio of patients is 63:2. This male preponderance of 97% cannot be explained by different exposition rates. Distinct male preponderance is also noted in the neurotropic relative, *Cladophialophora bantiana* (G.S. de Hoog, unpub. data). Population 3 of *F. monophora* has a wider clinical spectrum than the remaining groups, comprising, in addition to chromoblastomycosis, several isolates from human brain infection. This population also comprised

some isolates from soil and plant debris acquired without use of mammal baits. Coexistence of closely interrelated entities differing in pathogenicity and virulence seems likely in *Fonsecaea* spp., as was also suggested for black yeasts (A.H.G. Gerrits van den Ende et al., unpub. data).

Our data demonstrate that AFLP fingerprinting is a tool that produces highly reproducible results for molecular epidemiology. The use of AFLP showed that local *Fonsecaea* agents of chromoblastomycosis seem able to be maintained over 70 years, and therefore epidemiologic profiles take the structure of expanding clones. By locality, patients are infected by only a limited number of genotypes. The fungi disperse slowly, leading to appreciable geographic structuring, which ultimately may lead to allopatric speciation (diversification resulting from geographic barriers). Few environmental strains have been recovered during repeated isolation experiments, whereas *Fonsecaea* spp. accumulates substantially in the human host. The mechanisms behind their pathology remain unexplained.

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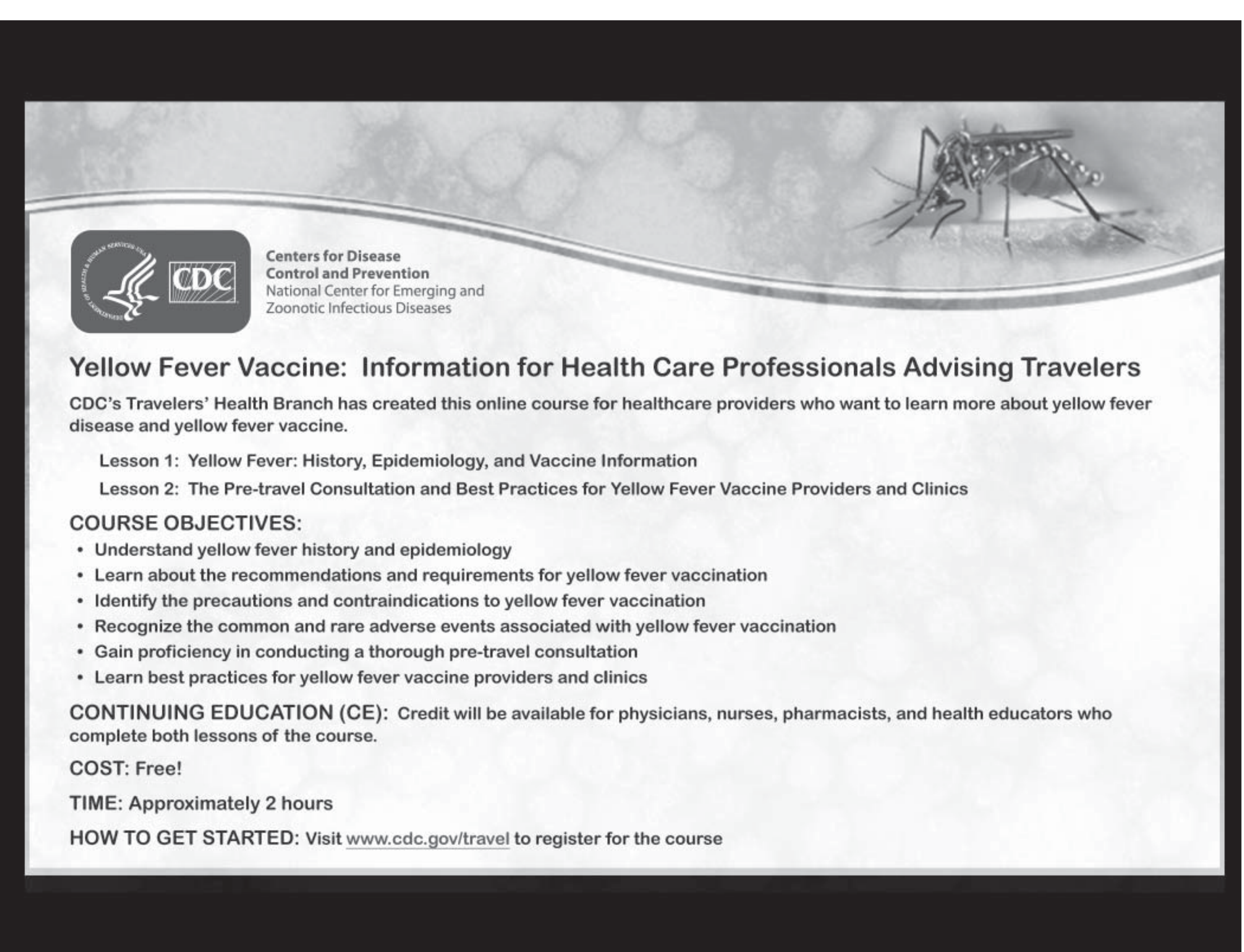

Monitoring and Characterization of Oseltamivir-Resistant Pandemic (H1N1) 2009 Virus, Japan, 2009–2010

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To monitor and characterize oseltamivir-resistant (OR) pandemic (H1N1) 2009 virus with the H275Y mutation, we analyzed 4,307 clinical specimens from Japan by neuraminidase (NA) sequencing or inhibition assay; 61 OR pandemic (H1N1) 2009 viruses were detected. NA inhibition assay and M2 sequencing indicated that OR pandemic (H1N1) 2009 virus was resistant to M2 inhibitors, but sensitive to zanamivir. Full-genome sequencing showed OR and oseltamivir-sensitive (OS) viruses had high sequence similarity, indicating that domestic OR virus was derived from OS pandemic (H1N1) 2009 virus. Hemagglutination inhibition test demonstrated that OR and OS pandemic (H1N1) 2009 viruses were antigenically similar to the A/California/7/2009 vaccine strain. Of 61 case-patients with OR viruses, 45 received oseltamivir as treatment and 10 received it as prophylaxis, which suggests that most cases emerged sporadically from OS pandemic (H1N1) 2009, due to selective pressure. No evidence of sustained spread of OR pandemic (H1N1) 2009 was found in Japan; however, 2 suspected incidents of human-to-human transmission were reported.

In March and early April of 2009, a new swine-origin A/H1N1 influenza virus, now called pandemic (H1N1) 2009, emerged in Mexico and the United States and spread rapidly (*1–3*). On June 11, 2009, the World Health Organization (WHO) declared a phase-6 pandemic alert, indicating a global pandemic. The earliest virus isolates were sensitive to the neuraminidase inhibitors (NAIs) zanamivir and oseltamivir, but resistant to M2 inhibitors, such as amantadine and rimantadine (*1,3–5*). Thus, the NAIs have been used globally for treatment and prophylaxis of pandemic (H1N1) 2009 virus inflection.

Oseltamivir-resistant (OR) pandemic (H1N1) 2009 was first detected in Japan, Denmark, and Hong Kong during May–June 2009 and has since been sporadically identified around the world (*6–8*). The OR pandemic (H1N1) 2009 viruses have a specific NA mutation, a histidine-to-tyrosine substitution at amino acid position 275 (N1 numbering, H275Y), that confers resistance to oseltamivir. In a report of 39 OR pandemic (H1N1) 2009 cases (as of October 22, 2009), 16 were associated with treatment, 13 were associated with postexposure prophylaxis, 3 were in NAI-untreated patients, and 7 were of unknown association (*8*). Preliminary global NAI surveillance showed 190 OR pandemic (H1N1) 2009 infections among >15,000 clinical specimens; thus, the global frequency of OR pandemic (H1N1) 2009 was <1.5% (as of January 8, 2010) (*9*). These reports indicated that human-to-human transmission of OR pandemic (H1N1) 2009 was limited but that oseltamivir treatment and prophylaxis could lead to emergence of OR pandemic (H1N1) 2009 virus.

A report for 1997–2007 showed that Japan accounted for ≈70% of the world's oseltamivir consumption (*10*). From August 2009 to March 2010, 9.76 million doses of

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oseltamivir were supplied in Japan, ≈2.3× that of the 2008–09 seasons (data from Chugai Co. Ltd, Tokyo, Japan). Thus, Japan is a high-risk environment for the development of OR pandemic (H1N1) 2009 virus because of drug use pressure. The emergence of such resistance is alarming, because OR seasonal influenza A (H1N1) viruses can rapidly spread worldwide once they acquire the capacity for human-to-human transmission (*11–15*). Additionally, in the 2009–10 season in Japan, almost all cases of influenza were caused by pandemic (H1N1) 2009 viruses (Figure 1). Thus, close surveillance must be maintained to detect pandemic (H1N1) 2009 and changes in its transmissibility and genetic and antigenic characteristics.

We monitored and characterized 4,307 clinical specimens collected in Japan during May 2009–February 2010 from patients with OR pandemic (H1N1) 2009 by NA sequencing, NAI assay, or both. Of them, we found 61 OR pandemic (H1N1) 2009 viruses with the H275Y mutation.

Materials and Methods

Virus Testing

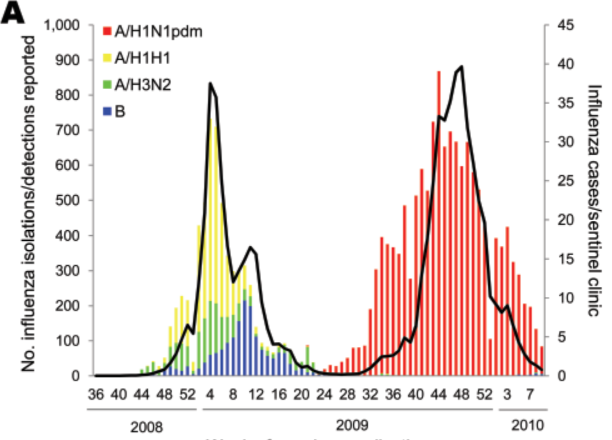
Influenza sentinel clinics and nonsentinel institutes send original samples to local public health laboratories for detection and virus isolation. In total, 4,307 clinical specimens, comprising both original samples (n = 440) and clinical isolates (n = 3,867), underwent either full or partial (nt 695–1110) NA sequencing to detect the H275Y mutation. Samples from 1,088 cases were collected before oseltamivir exposure, 516 were associated with oseltamivir use, 103 were associated with zanamivir use, and for 2,600, antiviral treatment status was unknown. We collected all OR pandemic (H1N1) 2009 isolates and randomly selected OS isolates (≈10%) from local public health laboratories. These representative OS and OR pandemic (H1N1) 2009 isolates underwent NA inhibition assay (421 OS and 61 OR viruses tested), full NA and hemagglutination (HA) sequencing (190 OS and 61 OR), internal gene (PB2/PB1/PA/NP/M/NS) sequencing (138 OS and 20 OR), and hemagglutination inhibition (HI) test (583 OS and 59 OR).

Sequence Analysis

Phylogenetic trees of NA and HA genes were constructed by neighbor-joining method. A phylogenetic tree was constructed by using representative OR and OS pandemic (H1N1) 2009 isolates from several prefectures of Japan. Sequence information of pandemic (H1N1) 2009 from other countries was downloaded from the Global Initiative on Sharing Avian Influenza Data (GISAID) and GenBank. All amino acid positions in the phylogenetic tree were described by N1 numbering.

NAI Assay

A chemiluminescent NAI assay was performed with the NA-star kit (Applied Biosystems, Tokyo, Japan) (*13*). Briefly, final drug concentration was 0.03–6,500



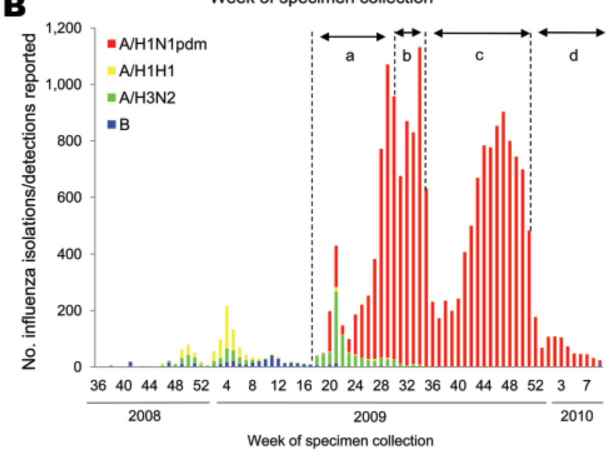


Figure 1. Weekly cases of influenza and isolation or detection of influenza viruses by influenza sentinel clinics (A) and nonsentinel clinics (B) from week 36 of 2008 to week 9 of 2010 in Japan (as of March 9, 2010). Pandemic (H1N1) 2009 (A/H1N1pdm) surveillance in Japan was divided into 4 stages depending on the prevalence situation, as shown in panel B: a) case-based surveillance (April 28–July 23), b) outbreak and hospitalization surveillance (July 24–August 24), c) hospitalization surveillance (August 25–December 20), and d) severe/fatal case surveillance (December 21 onwards). The sentinel clinics, consisting of 3,000 pediatric clinics and 2,000 internal medical clinics, collected samples randomly, while the nonsentinel clinics collected samples depending on the surveillance stage. Local public health laboratories randomly selected these samples for neuraminidase (NA) surveillance from both sentinel and nonsentinel clinics. In this study, 4,307 clinical specimens, comprising both original samples (n = 440) and isolates (n = 3,867), were subjected to full or partial NA sequencing for detection of the H275Y mutation. All oseltamivir-resistant (n = 61) and ≈10% of oseltamivir-susceptible pandemic (H1N1) 2009 (n = 421) isolates were then subjected to NA assay. The treatment history of the 4,307 cases consists of NA inhibitor–untreated (n = 1,088), oseltamivir use (n = 516), zanamivir use (n = 103), and unknown history (n = 2,600). Black line in panel A indicates weekly cases of influenza-like illness per influenza sentinel clinic.

nM for oseltamivir and 0.03–12,500 nM for zanamivir. Chemiluminescence was assayed with an LB940 plate reader (Berthhold Technologies, Bad Wilbad, Germany). Drug concentrations required for 50% inhibitory concentration of NA activity (IC_{50}) were calculated with MikroWin 2000 software (ver. 4; Mikrotek Laborsysteme GmbH, Overath, Germany). To validate the NAI assay, we used already characterized drug-resistant viruses and sensitive counterparts as controls: A/Hokkaido/15/2002 (155H) and A/Hokkaido/9/2002 (155Y), zanamivir (*16*); A/Denmark/528/2009pdm (275Y), A/Denmark/524/2009pdm (275H), seasonal-H1N1 A/Yamagata/68/2008 (275Y), A/Yamagata/41/2008 (275H), oseltamivir.

Statistical Analyses

Box-and-whisker plots were used to determine the cutoff value between NAI-resistant (outlier) and -sensitive viruses. The box contains 50% of the results, representing the middle 2 quartiles (25%–75%). The length of the box shows the interquartile range (IQR). The cutoff value was defined as the upper quartile + 3.0 × interquartile range from the 25th to 75th percentile. For statistical analyses, OR pandemic (H1N1) 2009 viruses with the H275Y mutation were excluded from the overall population.

HI Test

An HI test was performed to evaluate the reactivity of ferret antiserum against the 2009/10 vaccine strain A/California/7/2009, as described in the WHO Manual (*17*). The efficacy of ferret postinfection antiserum against egg-grown A/California/7/2009 was used as a reference. Antiserum was treated with receptor-destroying enzyme II (Denka Seiken, Tokyo, Japan) and adsorbed with turkey erythrocytes before testing, to prevent nonspecific reactions. A 0.5% suspension of turkey erythrocytes was used for the HI test.

Results

Geographic Distribution of OR Pandemic (H1N1) 2009

The 4,307 clinical specimens isolated during May 2009–February 2010 were collected from 41 of 47 prefectures in Japan, and the H275Y mutation was detected by NA sequencing. In total, 61 (1.4%) OR pandemic (H1N1) 2009 viruses possessed the H275Y (n = 48) or 275H/Y mixed (n = 13) mutations (Figure 2). OR pandemic (H1N1) 2009 emerged sporadically in several prefectures and was detected over a period of several months (Figures 2, 3).

Patient Treatment History and Epidemiologic Background

Of 4,307 case-patients, 516 had oseltamivir treatment, 103 had zanamivir treatment, 1,088 were NAI-untreated, and the treatment history of 2,600 was unknown. Of the 61 cases of OR pandemic (H1N1) 2009, 45 were associated with oseltamivir treatment, 10 with postexposure prophylaxis, and 6 occurred in NAI-untreated patients; thus, oseltamivir treatment and prophylaxis likely accelerated emergence of OR pandemic (H1N1) 2009. The relationship between time of sampling (days after oseltamivir treatment) and OR pandemic (H1N1) 2009 detection showed that OR pandemic (H1N1) 2009 was generally detected at least 4 days after oseltamivir treatment (Table 1).

Figure 2. Geographic distribution of H275Y-harboring oseltamivir-resistant pandemic (H1N1) 2009 viruses in Japan, May 2009–February 2010. Values are no. oseltamivir-resistant isolates/total no. tested. Overall prevalence in Japan was 1.4% (61/4,307).

Of the 61 case-patients, 36 (59%) were male; 19 (31%) were 0–4 years, 25 (41%) were 5–18 years, 12 (20%) were 19–50 years, and 5 (8%) were >50 years. Underlying medical conditions were known for 41; 24 had chronic underlying medical conditions (pulmonary [13], neurologic [4], blood [3], diabetes [1], kidney disease [1], immunocompromised [5], other conditions [2]).

Two Possible Cases of Human-to-Human Virus Transmission

Almost all OR pandemic (H1N1) 2009 cases emerged sporadically and were not epidemiologically linked. However, 2 cases of human-to-human transmission were suspected. One was observed in Niigata Prefecture where 2 children hospitalized in the same room were infected with OR pandemic (H1N1) 2009 virus within a few days. Symptoms developed first in a 4-year-old girl on October 10,

2009, and she received oseltamivir. OR pandemic (H1N1) 2009 virus was isolated from this patient on October 14. A 6-year-old boy in the same room received prophylaxis (by treatment dosage) with oseltamivir beginning October 10. However, he experienced symptoms on October 13, and OR pandemic (H1N1) 2009 virus was isolated on October 14. The patients were in a double room, and patients with pandemic (H1N1) 2009 were not around them. Genetic analyses of the 2 viruses (i.e., A/Niigata/1233/2009 and A/Niigata/1234/2009) showed only 1 aa difference (D to G), at position 256 in PB2, and they also shared unique changes in NS1 and in PB1 (Table 2). Transmission possibilities were as follows: 1) OR pandemic (H1N1) 2009 was directly transmitted from the female patient or 2) an OS pandemic (H1N1) 2009 was transmitted from the female patient and an OR pandemic (H1N1) 2009 virus emerged in the male patient.

The other suspected instance of human-to-human transmission occurred in Tottori Prefecture. In a 9-year-old boy, symptoms developed on December 18, 2009, and OR pandemic (H1N1) 2009 virus was isolated from a sample collected on the same day, before oseltamivir use. However, the patient's 2 brothers were both infected with pandemic (H1N1) 2009 virus and had received oseltamivir since December 15. Although samples from these persons were not available, OR pandemic (H1N1) 2009 likely emerged in 1 patient and was transmitted to the other.

Case Unrelated to Oseltamivir Use

Detailed epidemiologic information was available for 2 of 6 persons with OR pandemic (H1N1) 2009 infections untreated by NAIs. Besides the case in Tottori Prefecture, another occurred in Oita Prefecture. The index patient had a mild cough beginning on July 12, and typical

Figure 3. Phylogenetic analysis of influenza pandemic (H1N1) 2009 viruses neuraminidase (NA) (A) and hemagglutinin (HA) genes (B). Most pandemic (H1N1) 2009 viruses possessed the amino acid substitutions S203T in HA and V106I and N248D in NA. Red, oseltamivir-resistant pandemic (H1N1) 2009 from Japan; green, oseltamivir-resistant pandemic (H1N1) 2009 from outside Japan; black, oseltamivir-susceptible (OS) pandemic (H1N1) 2009; purple, 2009–10 current vaccine strains. The sampling month of each isolate is listed following the strain name. The phylogenetic tree of NA and HA genes was constructed by using the neighbor-joining method. Scale bars indicate nucleotide substitutions per site.

Table1. Relationship between detection of oseltamivir-resistant pandemic (H1N1) 2009 and interval from oseltamivir treatment to sample collection, Japan, 2009–2010*

	Days after oseltamivir treatment, N = 516†											
No. samples	Unknown	0‡	1	2	3	4	5	6	7	8	9	>10
Total no.	169	54	116	54	37	31	36	7	6	1	3	2
No. oseltamivir-resistant pandemic (H1N1) 2009	3	0	4	2	4 (1)	13 (1)	19 (1)	3 (2)	2	1 (1)	2 (2)	2 (2)

*Of total 4,307 specimens tested, neuraminidase inhibitor treatment history was available for 1,707; of these specimens, 516 were from patients who had received oseltamivir treatment.
†Parentheses indicate prophylactic use, e.g., 4 (1) = 1 of 4 total uses was for prophylaxis.
‡Day 0 represents the samples collected within 24 h after oseltamivir use.

influenza symptoms developed on July 15. OR pandemic (H1N1) 2009 virus was detected in a sample taken on July 16, before oseltamivir use. However, symptoms had developed in the index patient's son on July 11; the boy received zanamivir on July 12 (OR pandemic (H1N1) 2009 virus was not detected from a sample taken that day). No reports have indicated that zanamivir can induce OR virus with the H275Y mutation. The OR pandemic (H1N1) 2009 virus may have thus emerged naturally, with no selective pressure. However, the index patient may have been exposed to an oseltamivir-treated person outside of her household who harbored OR pandemic (H1N1) 2009 virus.

Genetic Analysis

Phylogenetic analyses of the HA and NA genes showed that most shared amino acid changes: S203T in HA and V106I and N248D in NA (Figure 3). In both trees, OR pandemic (H1N1) 2009 isolates were genetically scattered and possessed several sporadic amino acid changes, but each OR pandemic (H1N1) 2009 was genetically close to OS pandemic (H1N1) 2009 (Figure 3). Several OR pandemic (H1N1) 2009 isolates from Japan were also closely related to OR pandemic (H1N1) 2009 isolates from other countries.

Analysis of the genomes of representative OR (n = 20) and OS pandemic (H1N1) 2009 (n = 138) provided further insight into their similarities. First, comparison of the internal amino acid sequences of each OR pandemic (H1N1)

Table 2. Amino acid differences of each internal protein between oseltamivir-resistant (n = 20) and oseltamivir-sensitive (n = 138) pandemic (H1N1) 2009 virus, by strain, Japan, 2009–2010*

Strain	M1	M2	NP	NS1	NS2	PA	PB1†	PB2†
A/Niigata/1459/2009						V122I	**I435V**, N537S	
A/Osaka/2024/2009							**I435V**	R251K
A/Shimane/1062/2009						V127A, T357I	**I435V**	N448S
A/Shimane/188/2009				A102T			**I435V**	R54K
A/Yokohama/1340/2009						S186N	**I435V**	
A/Yokohama/1394/2009							**I435V**, F466Y	
A/Shiga/61/2009			V119I	M93I			**I435V**, A93V, T257A	**K660R**
A/Niigata/1233/2009				M93I, E217K			T257A	**K660R**
A/Niigata/1234/2009				M93I, E217K			T257A	**K660R**, D256G
A/Shiga/45/2009								**K660R**
A/Chiba/1017/2009								
A/Iwate/3/2009	K103R‡							
A/Mie/100/2009		S23N		T94N			V609A	R251K
A/Oita/126/2009			I100V	E55G, V103I		L370I	K480R	
A/Osaka/180/2009	A33T			V103I	E63K		I667T	V649I, E700K
A/Saitama-C/88/2009				E208K	M50I	A70V		V227I
A/Sapporo-C/190/2009		D21G						
A/Shiga/43/2009							A652T	
A/Tokushima/2/2009						M311I		
A/Yamaguchi/22/2009						V379I		

*M, matrix protein; NP, nucleoprotein; NS, nonstructural protein; PA, polymerase A; PB, polymerase B.
†Of the 138 oseltamivir-sensitive pandemic (H1N1) 2009 virus samples, I435V and K660R (**boldface**) were observed from 32 and 12 isolates, respectively. These changes would sporadically occur in both pandemic (H1N1) 2009 isolate types.
‡Lys (K) at position 103 in M1 protein, consensus amino acid among the oseltamivir-resistant pandemic (H1N1) 2009 virus, was replaced with Arg (R).

2009 and OS pandemic (H1N1) 2009 isolate consensus showed that OR viruses possessed several sporadic amino acid changes, but did not exhibit any common amino acid changes unique to OR pandemic (H1N1) 2009 viruses, indicating that the internal genes of OR and OS pandemic (H1N1) 2009 viruses were genetically indistinguishable (Table 2). Second, comparison of a 2 samples from a patient with pandemic (H1N1) 2009 before and after oseltamivir treatment (A/Chiba/1016/2009 and A/Chiba/1017/2009) showed only the H275Y change in NA and no changes in any other proteins. Finally, no evidence of reassortment of pandemic (H1N1) 2009 and seasonal influenza A (H1N1) viruses was detected.

Of 61 pandemic (H1N1) 2009 OR isolates, those from 13 patients were of mixed NA gene populations (H275 and Y275). Because all 13 patients had received oseltamivir, these samples would have been collected during selective pressure–induced generation of OR pandemic (H1N1) 2009 from OS pandemic (H1N1) 2009 (online Appendix Figure, www.cdc.gov/EID/content/17/3/470-appF.htm). Because calculating precise IC_{50} values from a mixed population of NAI-resistant and -sensitive viruses is not possible (*13,18*), the 13 mixed isolates were excluded from the overall population for the purposes of the statistical analysis of OR.

Antiviral Drug Susceptibility

NAI data are summarized in Table 3. The average IC_{50} value of OR pandemic (H1N1) 2009 (n = 48) for oseltamivir was 370-fold higher than that of OS pandemic (H1N1) 2009 (n = 421) viruses. For zanamivir, 3 of 482 viruses were identified as outliers (cutoff >0.60 nM). Compared with the consensus sequence of OS pandemic (H1N1) 2009, one OS pandemic (H1N1) 2009 A/Okayama/17/2009pdm (0.61 nM) had a D151D/N mixture in its NA protein, and 2 OR pandemic (H1N1) 2009, A/Shiga/43/2009pdm (0.64 nM) and A/Yokohama/1538/2009pdm (0.64 nM) possessed I34V and I195V substitutions in the NA protein, respectively (online Appendix Table, www.cdc.gov/EID/content/17/3/470-appT.htm). The IC_{50} values of OS and OR pandemic (H1N1) 2009 viruses were similar to those of their seasonal influenza A (H1N1) counterpart viruses (Table 3).

Susceptibility to M2 inhibitors was determined by M2 sequencing. All tested viruses, including OR (n = 20) and OS pandemic (H1N1) 2009 (n = 138), had an S31N resistance marker in the M2 protein, suggesting that all pandemic (H1N1) 2009 isolates were resistant to M2 inhibitors.

Antigenic Characterization

The HI test was performed to estimate the reactivity of OS (n = 583) and OR pandemic (H1N1) 2009 (n = 59) virus to ferret antiserum against the 2009–10 vaccine strain A/California/7/2009. More than 93% of OS (n = 546) and OR pandemic (H1N1) 2009 (n = 55) isolates were inhibited by anti-A/California/7/2009 ferret antiserum, and 5.8% and 5.1% of OS (n = 34) and OR pandemic (H1N1) 2009 (n = 3), respectively, showed a 4-fold reduced HI titer. Only 0.5% and 1.7% of OS- (n = 3) and OR pandemic (H1N1) 2009 (n = 1), which had either the K153E or G155E changes in deduced antigenic sites in HA protein, showed at least an 8-fold reduction in HI titer. Thus, OS and OR pandemic (H1N1) 2009 are antigenically indistinguishable and similar to the 2009–10 current vaccine strain A/California/7/2009.

Discussion

The data presented here provide no evidence of sustained spread of OR pandemic (H1N1) 2009 in Japan.

Table 3. Summary of neuraminidase inhibition assay of oseltamivir-resistant and oseltamivir-sensitive pandemic (H1N1) 2009 virus to oseltamivir and zanamivir*

Strain	IC_{50}, (nM/L)					
	Oseltamivir			Zanamivir		
	No. isolates	Mean ± SD (range)	Cutoff value	No. isolates	Mean ± SD (range)	Cutoff value
Pandemic (H1N1) 2009						
Oseltamivir-sensitive	421	0.10 ± 0.02 (0.05–0.19)	>0.20	421	0.28 ± 0.06 (0.11–0.61)	>0.60†
Oseltamivir-resistant	48‡	37.28 ± 14.06 (20.69–80.91)	NC	61	0.36 ± 0.11 (0.17–0.64)	
Seasonal influenza (H1N1) (A/Yamagata/41/2008)						
Oseltamivir-sensitive		0.09 ± 0.02§			0.24 ± 0.10	
Oseltamivir-resistant		51.76 ± 9.54			0.37 ± 0.13	

*IC_{50}, 50% inhibitory concentration; NC, not calculated.
†Because both IC_{50} values of OS and OR pandemic (H1N1) 2009 viruses were indistinguishable, the cutoff values for zanamivir were calculated from the overall population (N = 482).
‡IC_{50} values of 13 mixed samples with H275 and Y275 were excluded from overall population in statistical analysis of OR isolates.
§Mean ± SD IC_{50} values of control seasonal influenza A (H1N1) viruses were determined from 10 independent experiments for oseltamivir and 2 for zanamivir.

In this study, clinical specimens were collected from both NAI-untreated and NAI-treated patients, so later samples were collected after the exertion of selective pressure by drug treatment. However, frequency of detection of OR pandemic (H1N1) 2009 was low (1.4%). Because OR and OS pandemic (H1N1) 2009 isolates were genetically and antigenically indistinguishable, the current 2009–10 vaccine would be expected to be effective against recent OR pandemic (H1N1) 2009. No evidence of reassortment with seasonal influenza A (H1N1) virus was detected. Immunocompetent patients infected with OR pandemic (H1N1) 2009 showed typical uncomplicated influenza symptoms, similar to those caused by OS pandemic (H1N1) 2009 (*19*).

Early reports suggested that ≈70% of the worldwide consumption of oseltamivir occurs in Japan (*10*), but long-term NAI surveillance in Japan from 1996 to 2007 (*10*) and previous NAI surveillance (*16,20,21*) showed a low frequency of resistant viruses, suggesting that the transmissibility of OR viruses selected by drug pressure was remarkably reduced. However, beginning in November 2007, an unexpectedly high frequency of OR seasonal influenza A (H1N1) viruses with the H275Y mutation was detected in Europe (*11–15*). Most were isolated from NAI-untreated patients and were more transmissible than OS influenza A (H1N1), resulting in rapid global dissemination (*15*). In contrast, even in the 2007–08 season, OR influenza A (H1N1) was detected only rarely (1.5%–2.6%) in Japan, despite the high level of oseltamivir use (*13,22*). However, OR influenza A (H1N1) virus was detected at a far higher frequency (≈100%) the next year (*13*). Thus, the pattern of oseltamivir use did not correspond to the emergence and widespread distribution of OR influenza A (H1N1) viruses.

In contrast, this study and a recent report (*8*) found that OR pandemic (H1N1) 2009 has been detected predominantly in isolates from oseltamivir recipients. Unlike recent OR influenza A (H1N1) viruses, such OR pandemic (H1N1) 2009 viruses seemed to have restricted transmissibility among humans. These findings indicated that oseltamivir use was responsible for the emergence of OR pandemic (H1N1) 2009 viruses, but perhaps not for the widespread distribution of OR pandemic (H1N1) 2009.

Although the reason why recent OR seasonal influenza A (H1N1) isolates did not lose fitness remains unclear, a functional defect in NA proteins caused by H275Y may be counteracted by permissive secondary mutations. Two such mutations, R222Q and V234M, have been identified in seasonal influenza (H1N1) (*23*). Although whether the amino acids of the corresponding positions of pandemic (H1N1) 2009 play a similar role is unknown, the NA protein of this virus does have A and V residues at positions 222 and 234, respectively; a V at position 234 was identical to that in a nonpermissive amino acid sequence. Other sporadic and some shared amino acid change(s) were observed in the NA protein of OR pandemic (H1N1) 2009 viruses, but these changes apparently did not restore viral fitness, because no efficiently transmissible OR pandemic (H1N1) virus was found. These observations suggest that the NA proteins of recent OR pandemic (H1N1) 2009 isolates likely did not possess such permissive secondary mutation(s) (online Appendix Table).

However, all recent animal studies of OR pandemic (H1N1) 2009 virus have shown that viral fitness and transmissibility did not differ from those of OS pandemic (H1N1) 2009 virus (*24–26*) and had a potential to supersede OS pandemic (H1N1) 2009 virus. Nevertheless, OR pandemic (H1N1) 2009 did not supersede OS pandemic (H1N1) 2009 in humans. This inconsistency may be explained by differences in infectious dose used in the animal models.

In an NAI assay of zanamivir susceptibility, statistical analysis identified 3 outliers. One OS pandemic (H1N1) 2009 possessed the D151D/N mutation, which has been reported to affect susceptibility to zanamivir in seasonal viruses (*20,21*). Two OR pandemic (H1N1) 2009 isolates had the substitutions I34V and I195V in NA; however, whether these affect zanamivir susceptibility is unclear. We also assayed peramivir susceptibility in representative OR and OS pandemic (H1N1) 2009 isolates. Data suggested that OR pandemic (H1N1) 2009 virus, which contained the H275Y substitution, possessed cross-resistance to peramivir, as reported by another group (*27*).

Both case reports and preliminary NAI surveillance (*8,19,28–32*) have indicated 2 groups are at high risk for the generation of resistant viruses. The first is patients with severely compromised or suppressed immune systems, who shed virus for prolonged periods and thus have an increased chance of developing resistant virus (*33,34*). WHO reported that 25% of 285 resistant cases (as of April 17, 2010) occurred in immunocompromised patients (*35*). The second group is persons who are receiving postexposure prophylaxis, who take a subtherapeutic dose of 75 mg 1×/day (treatment dosage is 75 mg 2×/day). This regimen may only partially inhibit viral replication, thus facilitating the emergence of OR pandemic (H1N1) 2009. WHO recommends chemoprophylaxis only for persons who have a higher risk for severe or complicated illness (*19*). These groups were observed in our study.

We found that most OR pandemic (H1N1) 2009 virus was detected in samples collected at least 4 days after oseltamivir treatment or prophylaxis (Table 1). However, the frequency of OR pandemic (H1N1) 2009 in each day of treatment with oseltamivir could not be calculated because of a lack of treatment history data. This timing is consistent with that for OR seasonal influenza A (H1N1, H3N2) and pandemic (H1N1) 2009 viruses, which typically emerge

3–6 days after oseltamivir treatment (*36–38*). Additionally, we also observed the rapid emergence of OR pandemic (H1N1) 2009 virus within 48 hours of oseltamivir exposure (*39*). Nontheless, 6 cases occurred in untreated patients. Indeed, the OR pandemic (H1N1) 2009 in the Oita case may likely be a natural occurrence. We are aware of only 2 other reports, one from Vietnam and one from Hong Kong, of naturally occurring OR pandemic (H1N1) 2009 in untreated patients (*7,40*).

The greatest concern regarding OR pandemic (H1N1) 2009 is that drug-resistant viruses will acquire the ability to be transmitted efficiently among humans as has recent OR seasonal influenza A (H1N1). Two hospital outbreaks in the United Kingdom and the United States have been reported (*31,32*). In both, the immune systems of all patients were severely compromised or suppressed, indicating that these patients had an increased risk for not only the emergence of OR pandemic (H1N1) 2009 virus, but also OR seasonal influenza (H1N1) virus (*31,32*). In contrast, particular attention should be paid to the Vietnamese case because a naturally occurring OR pandemic (H1N1) 2009 virus caused a cluster of 7 cases in immunocompetent patients with no history of oseltamivir use (*40*).

Despite the high level of oseltamivir use in Japan, prevalence of OR pandemic (H1N1) 2009 remains low (1.4%). Thus, oseltamivir remains the first option for treating pandemic (H1N1) 2009, but zanamivir should be considered for immunocompromised patients. Additionally, as first priority for prophylaxis of both OR and OS pandemic (H1N1) 2009 infection should be vaccination, but not antiviral agents. Conversely, a preclinical animal model showed that OR pandemic (H1N1) 2009 had high potential to acquire transmissibility without losing viral fitness (*24–26*). Whether and how OR pandemic (H1N1) 2009 may acquire efficient transmissibility among humans are not known. Thus, vigilant monitoring of OR pandemic (H1N1) 2009 infection and alterations in its transmissibility and antigenic and genetic characteristics is essential.

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Novel Picornavirus in Turkey Poults with Hepatitis, California, USA

Kirsi S. Honkavuori, H. L. Shivaprasad, Thomas Briese, Craig Street, David L. Hirschberg, Stephen K. Hutchison, and W. Ian Lipkin

To identify a candidate etiologic agent for turkey viral hepatitis, we analyzed samples from diseased turkey poults from 8 commercial flocks in California, USA, that were collected during 2008–2010. High-throughput pyrosequencing of RNA from livers of poults with turkey viral hepatitis (TVH) revealed picornavirus sequences. Subsequent cloning of the ≈9-kb genome showed an organization similar to that of picornaviruses with conservation of motifs within the P1, P2, and P3 genome regions, but also unique features, including a 1.2-kb sequence of unknown function at the junction of P1 and P2 regions. Real-time PCR confirmed viral RNA in liver, bile, intestine, serum, and cloacal swab specimens from diseased poults. Analysis of liver by in situ hybridization with viral probes and immunohistochemical testing of serum demonstrated viral nucleic acid and protein in livers of diseased poults. Molecular, anatomic, and immunologic evidence suggests that TVH is caused by a novel picornavirus, tentatively named turkey hepatitis virus.

Turkey viral hepatitis (TVH) is a highly infectious disease affecting young turkey poults. The disease is often subclinical, causing minor histologic lesions, and becomes overt when the animals are stressed, resulting in varying rates of illness and death (*1*). Mortality rates of up to 25% have been reported (*2*). Diagnosis is based on characteristic lesions in the liver, which include multifocal necrosis and mononuclear inflammatory cell infiltrates (*3*,*4*). Similar lesions may be found in the pancreas. Clinical signs include anorexia, depression, diarrhea, and weight loss compatible with a diagnosis of enteritis, the second most common diagnosis made in turkey poults throughout the United States. Although we cannot with confidence estimate the specific burden of TVH, its economic effects are likely substantial; in the United States, turkey production was valued at $3.71 billion in 2007. The identification of a pathogen and development of specific diagnostics will lead to better understanding of the economic consequences and other effects of TVH.

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The disease has been experimentally reproduced in turkey poults by inoculation with material derived from affected animals (*1–4*). A viral basis for TVH has been presumed since its initial description in 1959 because the causative agent passed through 100-nm membranes, was acid stable, was not affected by antimicrobial drugs, and could be propagated in the yolk sac of embryonated chicken eggs (*3–5*). Icosahedral particles of 24 to 30 nm have been found by electron microscopy (EM) in liver lesions of birds (*6*) as well as in embryonated turkey eggs (*1*) that have been inoculated with material derived from affected birds; however, no agent has been consistently implicated (*7,8*).

Materials and Methods

Animals

Samples from healthy and diseased turkey poults were collected from February 2008 through January 2010 from 8 commercial flocks in California, USA. Flock sizes ranged from 22,500 to 40,000 birds. Clinical signs in diseased poults included anorexia, lethargy, diarrhea, and increased mortality rates. Postmortem analyses revealed livers with white foci and, occasionally, pale patchy areas in the pancreas. Histopathologic examination showed necrosis of hepatocytes and acinar cells of the pancreas and inflammation.

High-throughput Pyrosequencing

Total RNA was extracted from 4 sick 25-day-old turkey poults by using TRI Reagent (Molecular Research Center, Inc. Cincinnati, OH, USA) and treated with DNaseI (Ambion, Austin, TX, USA). Two micrograms of DNaseI-treated RNA was reverse transcribed by using Superscript II (Invitrogen, Carlsbad, CA, USA) and random octamer primers with an arbitrary specific anchor sequence as described previously (*9*). cDNA was RNase H-treated before random amplification by PCR. The resulting products were purified by using MinElute (QIAGEN, Hilden, Germany) and ligated to linkers for sequencing on a GSL FLX Sequencer (454 Life Sciences, Branford, CT, USA). Sequences were clustered and assembled into contiguous fragments (contigs) after trimming of primer sequences, and BLAST analysis was applied to compare contigs (or single reads) at the nucleotide and amino acid levels to the GenBank database (www.ncbi.nlm.nih.gov).

PCR and Genome Sequencing

Primers were designed that bridged contigs identified by high-throughput sequencing. Additional primers were selected to resequence the genome with special attention to junctions of the P1 and P2 regions. The PCRs were conducted by using HotStar polymerase (QIAGEN) primers at 0.2 μmol/L each and 1 μL of random hexamer-primed cDNA. The draft genome sequence was confirmed by selecting additional primers to generate ≈1 kb products across the entire sequence for direct dideoxy sequencing (Genewiz, South Plainfield, NJ, USA), applying TaKaRa LA Taq polymerase with GC buffer (TaKaRa Bio, Otsu, Japan) to obtain products in areas that proved difficult to amplify because of potential secondary structures or elevated GC content.

Quantitative TaqMan Real-time PCR

Primers and probes for quantitative real-time PCR were selected within the 5′ untranslated region (UTR) of the turkey hepatitis virus (THV) genome by using Primer Express 1.0 software (Applied Biosystems, Foster City, CA). The primer/probe set THVforward1 5′-CACCCTCTAYGGGCAATGT-3′, THVreverse1 5′-TCAGCCAGTCTATGGCCAGG-3′, and THVprobe1 6FAM-5′-TGGATTCCCATCTCACGCGTCCAC-3′-TMR used in assay 1 (Table 1) was chosen on the basis of the initial THV strains sequenced. Primer THVforward2 5′-CACCCTYYAYGGGCAAATGT-3′ and probe THV probe2 6FAM-5′-ATTCCCATCTCACGCGTCCAC-3′-TMR were later selected to address sequence variation of additional strains and used with THVreverse1 primer in assay 2 (Table 2). A calibration standard for both assays was generated from strain 2993A by cloning a 571-nt genomic fragment into the pGEM-T Easy vector (Promega Corp., Madison, WI, USA). PCRs were pursued in triplicate by using a StepOnePlus Real-time PCR system (Applied Biosystems), and a standard cycling profile of 45 cycles in a volume of 25 μL containing random hexamer-primed cDNA, 300 nmol/L primer (each), and 200 nmol/L probe. Results were expressed as mean copy number per 300 ng total RNA.

Phylogenetic Analysis

Phylogenetic analyses were performed based on THV P1, 2C/3C/3D, and full polyprotein sequence excluding divergent aa 799–1199. Sequences were aligned to selected members of the *Picornaviridae* family by ClustalW and trees were constructed by using the Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.2 (*10*). A Jukes-Cantor model was applied to calculate distance, and statistical significance was assessed by bootstrap resampling of 1,000 pseudoreplicate datasets.

In Situ Hybridization

Viral probes and β-actin control probes were designed and applied according to the QuantiGene ViewRNA protocol by using branched DNA technology (Panomics, Fremont, CA, USA). Twenty viral probes, 17–28 nt long, were selected that cover 500 nt of target sequence in 2B/2C. Forty β-actin probes, 17–26 nt long, were selected covering 873 nt of mRNA sequence. Five-micrometer–thick paraffin-embedded tissue sections were fixed, permeabilized with protease, and hybridized with oligonucleotides conjugated

Table 1. Real-time PCR measurement of viral sequences in organ samples from turkey poults with turkey viral hepatitis compared with controls, California, USA, 2008–2010*

Poult no.	Age, d	Organ	C_t	Virus copies†
Infected				
2993A	25	Liver	17.12	5.66×10^7
2993B	25	Liver	17.02	6.22×10^7
2993C	25	Liver	26.98	9.0×10^3
2993D	25	Liver	17.93	2.8×10^7
0091.1	28	Liver	17.24	5.1×10
0091.2	28	Liver	17.69	3.43×10^7
0091.3	28	Liver	23.26	2.57×10^5
1813.1	26	Liver	23.48	2.12×10^5
1813.2	26	Liver	21.23	1.53×10^6
1813.3	26	Intestine	20.94	1.97×10^6
0690	30	Liver	34.92	$8.98 \times 10^{\circ}$
1999	29	Liver	28.44	8.42×10^3
		Pancreas	25.97	7.32×10^4
		Intestine	24.85	1.96×10^5
Control				
1621.1	42	Liver	>36‡	Negative
1621.2	42	Liver	>36‡	Negative
1621.3	42	Liver	>36‡	Negative

*C_t, cycle threshold.
†In 300 ng total RNA. Copy numbers were calculated on the basis of a standard curve generated from cloned target sequences.
‡C_t >36 was rated as negative on the basis of the highest dilution of standard representing 5 copies.

Table 2. Real-time PCR measurement of viral sequences in samples from turkey poults with turkey viral hepatitis compared with controls, California, USA, 2008–2010*

Poult no.	Age, d	Sample	C_t	Virus copies†
Infected				
2641.1	29	Cloacal swab	21.68	2.28×10^7
		Bile	31.44	7.58×10^4
		Serum	43.12	1.2×10^2
2641.2	29	Cloacal swab	>44‡	Neg
		Bile	32.5	4.04×10^4
		Serum	38.04	1.67×10^3
2641.3	29	Cloacal swab	>44‡	Neg
		Bile	28.60	3.84×10^5
		Serum	34.18	1.55×10^4
2641.4	29	Cloacal swab	30.6	1.21×10^5
		Bile	27.72	6.48×10^5
		Serum	41.06	3.06×10^2
2641.5	29	Cloacal swab	28.11	5.09×10^5
		Bile	32.8	5.84×10^4
		Serum	41.33	2.71×10^2
394.1	28	Cloacal swab	>44‡	Neg
		Serum	43.27	8.17×10^1
394.2	28	Cloacal swab	40.7	4.55×10^2
		Serum	>44‡	Neg
394.3	28	Cloacal swab	>44‡	Neg
		Serum	>44‡	Neg
394.4	28	Cloacal swab	29.62	2.14×10^5
		Serum	>44‡	Neg
394.5	28	Serum	>44‡	Neg
394.6	28	Serum	>44‡	Neg
394.7	28	Serum	>44‡	Neg
394.8	28	Serum	>44‡	Neg
394. 9	28	Serum	31.07	9.34×10^4
3302.1	39	Serum	36.38	3.08×10^3
3302.2	39	Serum	>44‡	Neg
3302.3	39	Serum	>44‡	Neg
3302.5	39	Serum	>44‡	Neg
Control				
2491.1	32	Cloacal swab	>44‡	Neg
2491.2	32	Cloacal swab	25.67	2.2×10^6
2491.3	32	Cloacal swab	>44‡	Neg
2491.4	32	Cloacal swab	>44‡	Neg
2491.5	32	Cloacal swab	>44‡	Neg
2491.6	32	Cloacal swab	34.74	1.11×10^4
407.1	39	Cloacal swab	>44‡	Neg
407.2	39	Cloacal swab	>44‡	Neg
407.3	39	Cloacal swab	>44‡	Neg
407.4	39	Cloacal swab	>44‡	Neg

*C_t, cycle threshold; neg, negative.
†In 300 ng total RNA. Copy numbers were calculated on the basis of a standard curve generated from cloned target sequences.
‡C_t >44 was rated as negative on the basis of the highest dilution of standard representing 5 copies.

to alkaline phosphatase. After incubation with FastRed substrate, the slides were counterstained with hematoxylin and mounted with coverslips by using Permount (Fisher Scientific, Pittsburgh, PA, USA). Images were acquired by using a Zeiss AX10 Scope AI, ProgRes digital microscope camera and Mac Capture Pro 2.6.0 software (Jenoptik, Jena, Germany).

Immunohistochemical Analysis

Glass slides with embedded tissues were heated at 56°C for 10 min and washed in citrus clearing agent (Fisher Scientific) to remove paraffin. The sections were rehydrated through graded alcohol solutions. Endogenous peroxidase activity was blocked by incubation in 0.3% H_2O_2 diluted in methanol for 30 min. Nonspecific binding was blocked by incubating sections in 10% goat serum in phosphate-buffered saline (PBS) for 1 h at 37°C. Serum specimens from poults with or without disease were added to the sections at 1:1,000 dilutions in PBS for overnight incubation at 4°C. After being washed in PBS, horseradish peroxidase–labeled goat anti-turkey immunoglobulin G (KPL, Gaithersburg, MD, USA) was added at a 1:250 dilution in PBS for 1 h at 37°C. After PBS washes, the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) was used, with 3, 3′-diaminobenzidine tetrahydrochloride as substrate (ImmPACT DAB kit; Vector Laboratories). The sections were counterstained with hematoxylin and dehydrated through graded alcohols. Finally, the slides were mounted with coverslips by using Permount (Fisher Scientific) and examined under a Zeiss AX10 Scope AI light microscope at ×40 magnification (Jenoptik).

Results

Identification of THV

Unbiased high-throughput pyrosequencing of RNA extracted from livers of 4 poults with TVH (animals 2993A, 2993B, 2993C, and 2993D; Table 1) yielded ≈63,100 sequence reads with a mean length of 285 nt. Seven contigs (average length of 674 nt, comprising 105 sequence reads) and 2 singletons (read lengths of 486 nt and 498 nt) that together yielded 3,182 nt of sequence were identified after primer trimming and assembly. Analysis at the nucleotide level was not informative; however, BLASTx analysis revealed significant similarity to picornavirus sequences at the amino acid level. The remainder of the genome was determined from RNA of poult 2993D by RT-PCR with primers linking the individual contigs and singletons (Figure 1). Applying additional primers in various genome regions, a second 9-kb genomic sequence was generated from another diseased poult (animal 0091.1). Sequence analysis showed that the second strain had the same genome organization as strain 2993D and 96.8% aa and 89.9% nt sequence identity (GenBank accession nos. HM751199 and HQ189775).

THV Genome Organization

The THV genome, comprising >9,040 nt and 2,813 aa, is larger than that of equine rhinitis B virus (genus *Erbovirus*), the largest known picornavirus genome (*11*). The length chiefly reflects the presence of a 1.2-kb

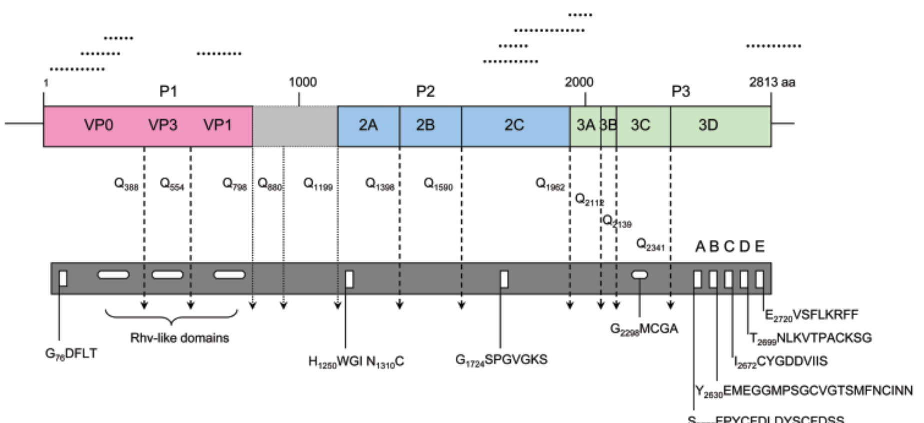


Figure 1. Predicted turkey hepatitis virus (THV) genome organization based on sequence comparison to known picornaviruses**.** Dotted lines above the genome depict the location of the original sequences obtained by high-throughput sequence analysis. Conserved picornaviral motifs and predicted potential cleavage sites along the coding region are indicated in the bar below.

sequence at the junction of the P1 and P2 regions in an otherwise typical picornavirus genome (Figure 1). The incomplete 461-nt 5′ UTR includes a 30-nt motif (nt 270–300 of THV) that is identical to duck hepatitis A virus 5′ UTR (*Avihepatovirus* genus), which has a type IV internal ribosome entry site. However, Mfold-modeling of the available sequence did not allow identification of the internal ribosome entry site type present in THV. At the 3′ end, 140 nt of the UTR were recovered (without reaching a poly-A tail) that showed no sequence similarity to other picornavirus 3′ UTRs.

With the exception of a highly conserved Gxxx[T/S] motif (*12*) that in some picornaviruses permits myristoylation, little sequence conservation occurs in the VP0 of THV with respect to other picornaviruses. Cleavage at an upstream Q_{73}/A would not generate an N terminal G, cleavage at this site is not supported by NetPicoRNA prediction, and the presence of a leader sequence is unclear (Figure 1; www.cbs.dtu.dk/services/NetPicoRNA). Therefore, as in parechoviruses, hepatitis A virus, and avian encephalomyelitis virus, this site is probably not functional for myristoylation in THV (*13–15*). NetPicoRNA analysis did not indicate a VP2/4 maturation cleavage as found in avihepatoviruses, kobuviruses, and parechoviruses. Sequence comparisons of the P1 region, mainly driven by recognizable sequence conservation in VP3 and a few regions in VP1, indicate the highest amino acid identity (20%) with turdiviruses, unclassified picornaviruses recently identified from wild birds (*16*). Homology in P1 to pfam sequence cluster cd00205 "picornavirus capsid protein domain like" (GenBank accession no. PF00073) was observed between amino acid residues 109–267, 391–539, and 622–768 (Figure 1). Potential cleavage sites within P1 are predicted after Q_{388} (VP0/3) and Q_{554} (VP3/1).

Protein 2A motifs in THV are conserved with respect to kobuviruses after a predicted cleavage site Q_{1199}. However, the sequence lacks the trypsin-like protease motifs that allow autocatalytic cleavage at the N-termini of enteroviruses and sapeloviruses, as well as the NPGP motif that facilitates C-terminal cleavage in aphthoviruses, avihepatoviruses, cardioviruses, erboviruses, senecaviruses, and teschoviruses. The predicted 2A sequence of THV resembles Hbox-NC motifs in hepatoviruses, kobuviruses, parechoviruses, and tremoviruses (H_{1250}WGI, N_{1310}C followed by a hydrophobic region L_{1332}-V_{1350}). The THV 2A protein may be generated by cleavage at conserved protease sites; however, multiple cleavage sites between P1 and P2 are predicted by NetPicoRNA (Figure 1). Cleavage after Q_{798} and Q_{1199} appears likely because it would generate VP1 and 2A products that align with other picornavirus proteins. As a result, 1 or 2 additional proteins (depending on cleavage at Q_{880}, Figure 1) may be produced from this genome region that have no homology to any viral product recorded in GenBank. Multiple 2A1, 2A2, or 2A3 protein products with undefined function are described in Ljungan virus, seal picornavirus, and duck hepatitis virus genomes (*17–19*). Although we predict, based on alignment analyses, that the C-terminal cleavage of 2A occurs at Q_{1398}, this prediction is poorly supported by NetPicoRNA. 2B and 2C have sequence homology to kobuvirus sequences, particularly in a conserved 2C helicase domain G_{1724}SPGVGKS that aligns to the PF00910 RNA helicase domain.

Although no sequence homology to any picornavirus record in GenBank was found for THV 3A, 3B displays a conserved tyrosine in position 3 as well as a conserved glycine in position 5. The THV 3C protease contains the active site motif G_{2298}MCGA, which is consistent with 3C proteases of other picornaviruses, and shows highest homology to cosaviral 3C (PF00548 3C cysteine protease [picornain] aligning to aa 2153–2321, Figure 1). The identity of a 472-aa sequence at the 5′ end of the genome as THV 3D is supported by homology of aa 2355–2809 to PF00680 RNA-dependent RNA polymerases, and the conservation of positive-strand viral RNA-dependent RNA polymerase motifs A–E (*20*) (Figure 1).

The assembled THV genome sequences were used to reanalyze the initial read library generated by unbiased high-throughput pyrosequencing. This analysis confirmed the presence, and overlap with, the adjacent sequences of divergent 2A and 3A region reads in the initial dataset.

Phylogenetic analyses based on amino acid sequence of the most informative genome regions 2C/3C/3D, the P1 region, and the full polyprotein sequence (excluding the nonconserved aa 799–1199) showed THV as a distinct species separate from classified genera (Figure 2). The 2C/3C/3D analysis indicates THV in an ancestral position to kobuviruses, klasseviruses, and turdiviruses, the viruses most related to THV.

THV RNA Load in Liver, Bile, Serum, and Cloacal Swab Specimens

Two TaqMan real-time PCR assays, both targeting the 5′ UTR, were developed to quantitate viral RNA load in affected animals. Assay 1 (Table 1) was replaced by assay 2 (Table 2) after the characterization of additional THV strains indicated divergent sequences.

In the liver samples from turkeys with TVH, viral RNA typically exceeded 10^5 copies/300 ng of total RNA; only 1 animal had a lower load (animal 0690, Table 1). No viral RNA was detected in livers from non-diseased control animals. Analysis of animals 1813.3 and 1999 indicated presence of the virus in the intestine and pancreas as well as in the liver. Cloacal swab samples, bile, and serum from 28-, 29- and 39-day-old poults with disease were also analyzed. Five of 9 cloacal swab specimens from TVH-affected animals were positive for viral RNA (Table 2). Viral RNA >10^4 copies/300 ng total RNA was detected in the bile of all 5 poults with TVH tested. Viral RNA was also found in serum samples from 8 of 18 poults with TVH.

Cloacal swabs were analyzed from 32- and 39-day-old turkey poults that did not have TVH according to histopathologic findings. Viral RNA was detected in 2 of 10 animals tested (Table 2).

Localization of THV RNA and Protein in Liver

The distribution of THV RNA was examined in liver sections of affected and unaffected poults by in situ hybridization. THV signal was found in the cytoplasm of hepatocytes of affected poults. No signal was observed in healthy poults (Figure 3). A hybridization signal with a control β-actin probe was present in both affected and healthy poults; however, β-actin signal was less pronounced in affected poults. In situ hybridization also indicated viral RNA in 1 intestinal sample from the 0091 animals (not shown), in line with the real-time PCR data obtained for animals 1813.3 and 1999 (Table 1).

Serum from TVH-affected poult 394.9, which was positive by PCR for THV RNA (Table 2), was used on paraffin-embedded tissues for immunohistochemical analysis. The serum showed reactivity when tested in livers from affected poults. No reactivity was observed with healthy poults (Figure 4).

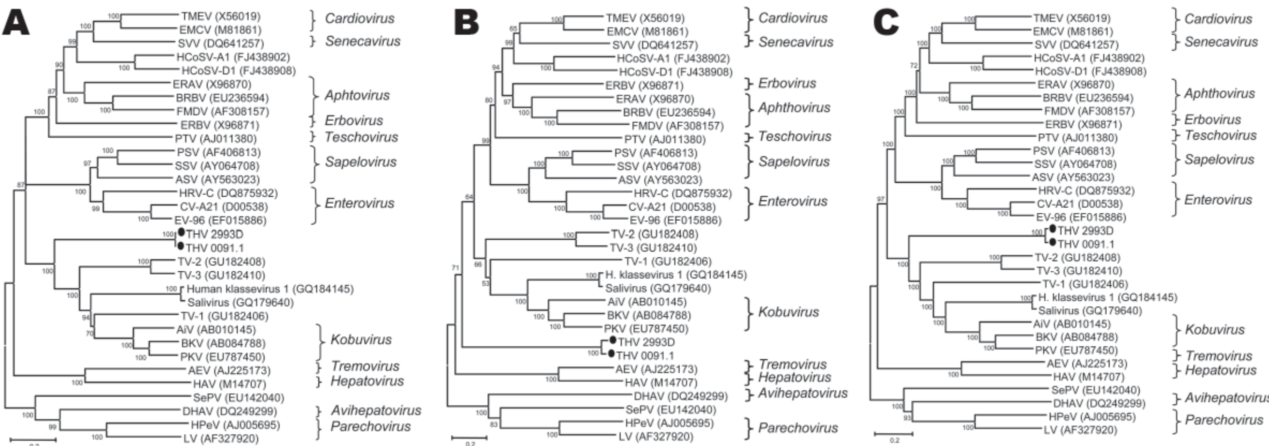


Figure 2. Relationships between turkey hepatitis virus (THV) and other picornaviruses. The phylogenetic analyses were based on amino acid sequences of the combined 2C, 3C, and 3D regions (A), the P1 region (B), and complete coding regions, excluding divergent aa 799–1199 (C). Representative sequences from different picornavirus genera and recently discovered, unclassified viruses were obtained from GenBank; accession numbers are indicated. Bootstrap values are given at the respective nodes; scale bars indicate number of amino acid substitutions per site. TMEV, Theiler's murine encephalomyelitis virus; EMCV, encephalomyocarditis virus; SVV, Seneca Valley virus; HCoSV-A1, human cosavirus A1; HCoSV-D1, human cosavirus D1; ERBV, equine rhinitis B virus; ERAV, equine rhinitis A virus; BRBV, bovine rhinitis B virus; FMDV, foot-and-mouth disease virus; PTV, porcine teschovirus; PSV, porcine sapelovirus; SSV, simian sapelovirus; ASV, avian sapelovirus; HRV-C, human rhinovirus C; CV-A21, coxsackievirus A21; EV-96, enterovirus 96; TV-2, turdivirus 2; TV-3, turdivirus 3; TV-1, turdivirus 1; AiV, Aichi virus; BKV, bovine kobuvirus; PKV, porcine kobuvirus; AEV, avian encephalomyelitis virus; HAV, hepatitis A virus; SePV, seal picornavirus; DHAV, duck hepatitis A virus; HPeV, human parechovirus; LV, Ljungan virus.

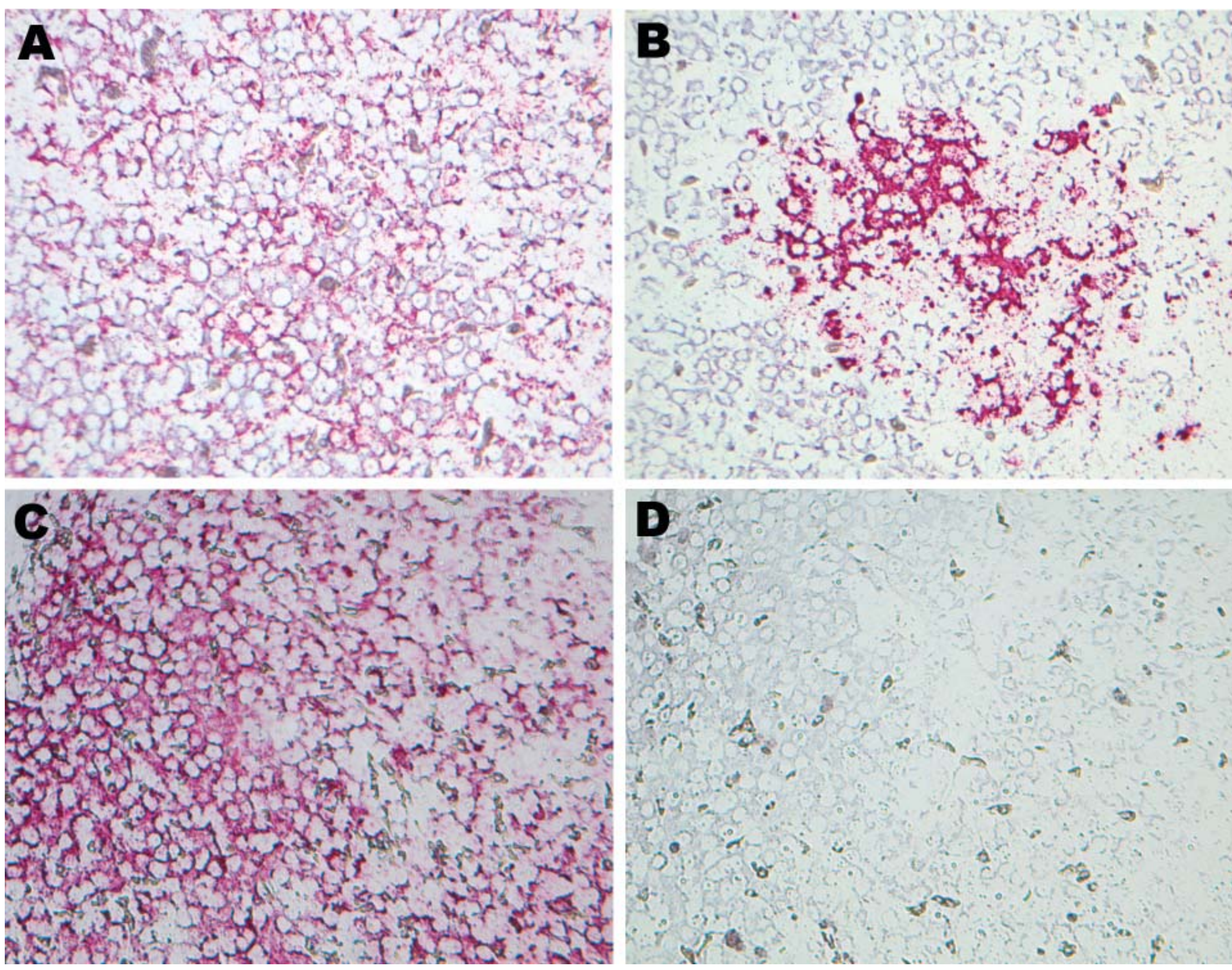


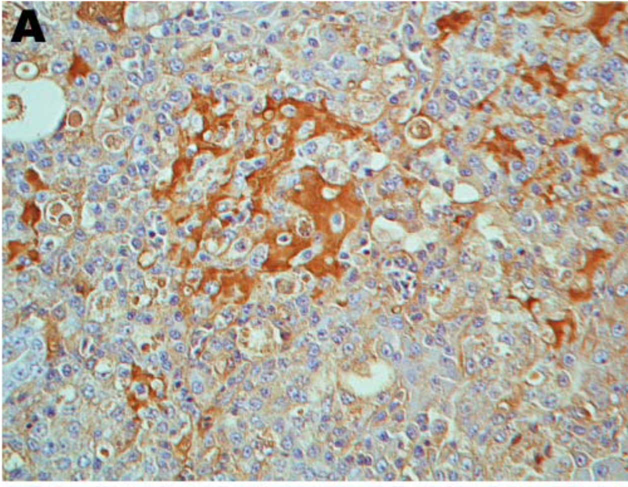
Figure 3. Results of in situ hybridization experiments. Hybridization of β-actin–specific and turkey hepatitis virus (THV)–specific oligonucleotide probes with FastRed staining on hepatitis-affected liver tissue from poult 2993A (A and B, respectively) and on nondiseased liver tissue from poult 1927B (C and D, respectively). Brightfield microscopy images; original magnification ×40.

Discussion

The *Picornaviridae*, a family of small, nonenveloped viruses with a positive-sense, single-strand RNA genome, currently consist of 12 genera. Recent additions to the family include salivirus NG-J1 and human klasseviruses identified in pediatric stool samples (*21–23*), cosaviruses (*24,25*), and an unclassified seal picornavirus 1 (*17*). Our phylogenetic analyses indicate that THV, although distantly related to viruses of the *Kobuvirus* genus as well as to unclassified klasseviruses/saliviruses and recently identified turdiviruses, is distinct from known picornaviruses.

Most peculiar is the unique 2A region of THV. THV appears to encode multiple 2A products. Although multiple 2A products have also been described for Ljungan virus, seal picornavirus, and duck hepatitis virus (*17–19*), the 2A region of THV shows no sequence homology to these products or to any other picornavirus sequence currently in GenBank. Also remarkable is the lack of sequence homology of THV 3A to picorna- or other virus sequences. Whereas VP3 (representing P1) is closest to turdivirus 1 (30% aa identity), 2B/C (representing P2) is closer to Aichi virus (28%) and 3C/D (representing P3) is closer to turdivirus 3 (39%). Thus, THV shows classic features of known picornaviruses but also unique features that do not support inclusion of THV into existing taxa of the *Picornaviridae* family.

Turkey poults with TVH may have diarrhea and pancreatitis as well as hepatitis. Picornavirus particles in the feces of animals with TVH have been described (*26,27*). Accordingly, we detected THV RNA in the intestine, pancreas, bile, and cloaca samples as well as the liver. These findings are consistent with a fecal–oral route for transmission. We also detected THV RNA in cloacal



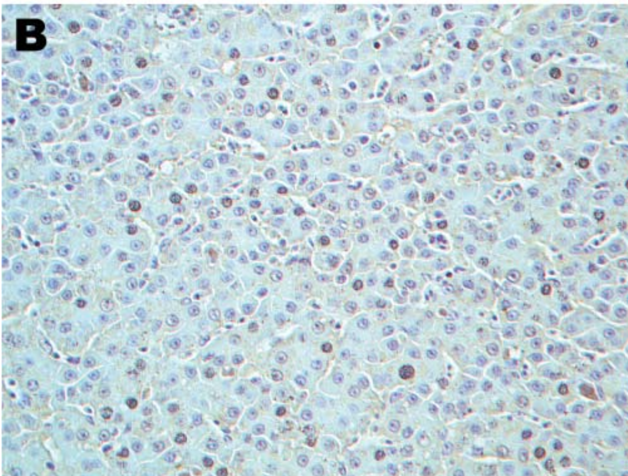


Figure 4. Immunohistologic staining of liver tissues with serum from a turkey poult with turkey viral hepatitis (TVH). Serum sample from PCR-positive poult 394.9 demonstrates turkey hepatitis virus (THV) antigens in clusters of cells in liver tissue of TVH-affected poult 2993A (A) but not in liver sections from nondiseased poult 1927B (B). Sections were counterstained with hematoxylin. Brightfield microscopy images; original magnification ×40.

swab samples from 2 of 10 asymptomatic poults. Because these animals were housed on a farm with history of TVH, this finding is consistent with reports suggesting subclinical infections (*2*,*6*). The advent of a noninvasive screening test for THV may aid in disease containment.

We molecularly characterized a picornavirus in turkey viral hepatitis and linked it to disease through measurements of load and tissue distribution, viremia and a humoral immune response to the agent. On the basis of the data presented here, we suggest that THV represents a new species in the order Picornavirales and a likely candidate for causing TVH.

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Tuberculosis among Health Care Workers

Iacopo Baussano, Paul Nunn, Brian Williams, Emanuele Pivetta, Massimiliano Bugiani, and Fabio Scano

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Learning Objectives

Upon completion of this activity, participants will be able to:

- Describe the risk for health care workers (HCWs) of developing incident latent tuberculosis infection (LTBI)
- Describe the risk for HCWs of developing tuberculosis (TB) disease
- Describe TB infection control measures that may be effective in healthcare settings

Editor

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To assess the annual risk for latent tuberculosis infection (LTBI) among health care workers (HCWs), the incidence rate ratio for tuberculosis (TB) among HCWs worldwide, and the population-attributable fraction of TB to exposure of HCWs in their work settings, we reviewed the literature. Stratified pooled estimates for the LTBI rate for countries with low (<50 cases/100,000 population), intermediate (50–100/100,000 population), and high (>100/100,000 population) TB incidence were 3.8% (95% confidence interval [CI] 3.0%–4.6%), 6.9% (95% CI 3.4%–10.3%), and 8.4% (95% CI 2.7%–14.0%), respectively. For TB, estimated incident rate ratios were 2.4 (95% CI 1.2–3.6), 2.4 (95% CI 1.0–3.8), and 3.7 (95% CI 2.9–4.5), respectively. Median estimated population-attributable fraction for TB was as high as 0.4%. HCWs are at higher than average risk for TB. Sound TB infection control measures should be implemented in all health care facilities with patients suspected of having infectious TB.

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Transmission of tuberculosis (TB) in health care settings to both patients and health care workers (HCWs) has been reported from virtually every country of the world,

regardless of local TB incidence. TB transmission occurs through droplet nuclei aerosolized by patients with infectious pulmonary TB and inhaled by other persons. Transmission is most likely to occur from unrecognized or inappropriately treated TB. The risk for transmission varies by setting, occupational group, local prevalence of TB, patient population, and effectiveness of TB infection control measures (*1*).

In resource-rich countries TB began to be recognized as an occupational hazard in the 1950s (*2*); since then, effective infection control measures have been implemented to reduce the risk for nosocomial TB (*3*). Poor implementation of infection control measures, the effects of the HIV epidemic on TB, and the emergence of multidrug-resistant (MDR) strains resulted in the reemergence of hospital-based transmission of TB and MDR TB in the early 1990s (*2*).

Most recently, the emergence of extensively drug-resistant (XDR) TB strains is posing a major public health threat in contexts characterized by a lack of proper infection control measures (*4*). Although the risk for latent TB infection (LTBI) and TB disease has generally been considered to be higher among HCWs than in the general population, some studies from countries with low TB incidence and others from countries with high TB incidence failed to show a higher estimated risk for TB among HCWs than among the corresponding local community.

We had 2 main objectives in conducting our study. First, by reviewing the literature, we assessed the rate of LTBI and relative risk and risk difference for the incidence of TB among HCWs worldwide, compared with the incidence of TB in the corresponding local general population. Second, we assessed the population-attributable fraction (PAF) (percentage) of TB in the general population associated with exposure to HCWs in their working settings.

Methods

Literature Search for Incidence among HCWs

An initial search of the literature for systematic reviews or reviews of meta-analyses estimated LTBI and TB incidence among HCWs worldwide allowed us to identify 4 systematic reviews (*1*,*5*–*7*). Most of the studies analyzed by Menzies et al. in 1995 were performed in the United States, United Kingdom, or Canada (*6*). In 2005, Seidler et al. focused on the risk for TB in low-incidence areas (*7*). In 2006, Joshi et al. analyzed low- and middle-income countries (*5*), and in 2007 Menzies et al. expanded their search and analyses, accounting also for high-income countries (*1*). We identified, retrieved, and considered for inclusion in our study all studies reported in the above-mentioned systematic reviews. To integrate and update the search, we searched 3 electronic databases for primary studies (i.e., PubMed, www.ncbi.nlm.nih.gov/pubmed; EMBASE, http://embase.com; and Web of Knowledge, www.isiwebofknowledge.com) to identify studies that assessed the incidence of LTBI and/or TB among HCWs published during January 2005–July 2010. The combination of terms adopted to perform the search is reported in the online Technical Appendix (www.cdc.gov/EID/content/17/3/488-Techapp.pdf). We restricted our search to articles published in English, French, Spanish, and Italian.

Study Selection

Studies were eligible for inclusion if they reported the incidence and the corresponding confidence intervals (CIs) of LTBI and TB disease among HCWs or if they reported the number of incident LTBI and TB cases identified in the study along with the overall number of HCWs investigated or the person-years of follow-up. LTBI incidence has been defined as tuberculin conversion after a documented negative-baseline tuberculin skin test (TST) as reported in the original study (*3*). We considered only studies adopting the intradermal TST technique. For cases of TB disease, we have included definitive (microbiologically confirmed) and presumptive (based on clinical, imaging, or pathology) diagnoses.

We excluded studies for a variety of reasons. Studies reporting only case series, only outbreak investigations, or only prevalence of LTBI and TB among HCWs were excluded, as were studies reporting investigations only of MDR TB and of only self-reported LTBI or TB. We also excluded studies that adopted interferon-γ release assay because of paucity of data and potential problems in pooling such data with those obtained from studies that adopted the TST technique. Furthermore, we excluded studies started before 1990 to decrease possible heterogeneity between studies associated with different awareness of TB as an occupational hazard for HCWs (*2*) and studies reporting data from studies investigating only HCWs working in outpatient clinics. All duplicate citations were eliminated from the initial database. Two of the authors (I.B., E.P.) screened these citations by reviewing titles and abstracts to identify potentially relevant studies and discussed disagreements until reaching a consensus. The database was then screened again to include only primary articles, and the full text of each citation was obtained and reviewed.

Data Extraction

Two of the authors (I.B., E.P.) designed a data extraction form. Then, all articles were independently reviewed and data extraction cross-checked.

The following data were collected from each study: country where the study was performed, study period, and health care setting under investigation. We also collected the

estimated incidence of LTBI and/or TB and corresponding CIs and/or number of incident LTBI and/or TB cases identified and the overall number of HCWs investigated or the person-years of follow-up. If reported, the incidence of LTBI and/or TB in a comparison group, such as the local general population or administrative workers not exposed to the health care setting under investigation, was also included. We used estimates provided by the World Health Organization (WHO) for the corresponding study period as estimates of TB incidence among the general populations in the host countries (*8*).

Data Collation and Meta-analysis

For each study, we considered the incidence for LTBI or TB among HCWs as reported in the original study. Adjusted estimates, when available, were preferred to crude estimates. Alternatively, we calculated the annual incidence of LTBI (hereafter referred to as annual risk for LTBI [ARTI]) and TB among HCWs. Then we estimated the incidence rate ratio (IRR), the risk difference of TB among HCWs compared with the corresponding general population, and the corresponding percentage of TB cases among HCWs attributable to exposure in health care settings. We used WHO estimates for the corresponding period (*8*) for TB incidence in the general population. Three studies could not be located and were excluded from the analysis.

We performed a random effects meta-analysis to account for the expected between-study variability for each study, i.e., we drew pooled estimates under the assumption that each study had different characteristics and measured different, though related, underlying yields (*9*). We used STATA version 11.0 software (StataCorp., College Station, TX, USA) for statistical analysis. The studies included in the meta-analysis were weighted by the inverse variance of their effect-size estimate (*10*).

To assess the fraction of TB in the population attributable to the exposure to health care settings, we calculated the PAF using Levin's formula (online Technical Appendix) (*11*). As a proxy for the proportion of the population working in health care settings, we considered the population of doctors, laboratory workers, nurses, and midwives as reported in WHO's Global Health Atlas (*8*). Community health workers were not included in this population because no study reported TB cases from these categories. We assessed heterogeneity across studies by the conventional χ^2 test for heterogeneity and by calculating the I^2 statistic, which accounts for the number of studies included in the meta-analysis and directly measures the variability not explained by information in the analysis (*12*).

To investigate possible sources of heterogeneity, we stratified the analysis accounting for TB incidence estimated in the country where the study had been conducted. In particular, we defined 3 strata: countries with low TB incidence (i.e., <50 cases/100,000 persons), countries with intermediate TB incidence (i.e., 50–99 cases/100,000 persons), and countries with high TB incidence (i.e., ≥100 cases/100,000 persons). Furthermore, to formally assess the effect of selected variables, such as the country income level as classified by the World Bank (*13*), the density of HCWs as reported by WHO (*8*), and the year the study was published, as possible sources of heterogeneity, we performed meta-regression analyses in which variance results from 2 additive components, 1 representing the variance within units, and the other the variance between units. Finally, we qualitatively assessed publication bias by drawing a funnel plot (online Technical Appendix Figure 1).

Results

We identified 846 potentially relevant unique citations from all literature searches and 74 from previous systematic reviews (Figure 1). Forty-three studies were eligible for inclusion (online Technical Appendix); these studies accounted for 1,176 cases of LTBI among HCWs and 49,576 person-years of follow-up and for 1,942 TB cases and >1,010,047 person-years of follow-up. Twenty-five and 18 studies reported LTBI and TB incident cases among HCWs, respectively (online Technical Appendix Tables 1, 2).

LTBI among HCWs

A median of 23 (interquartile range [IQR] 14–59) LTBIs occurred among HCWs. These LTBIs accounted for a median of 731 (IQR 111–2,144) person-years in each study.

The median estimated ARTI among HCWs was 2.9% (IQR 1.8%–8.2%) for studies from countries with low TB incidence, 8.7% (IQR 3.9%–10.5%) for studies from countries with intermediate TB incidence (in the present study only Brazil), and 7.2% (IQR 4.1%–14.3%) for studies from countries with high TB incidence. Consistently, the stratified pooled estimates for the ARTI were 3.8% (95% CI 3.0%–4.6%, I^2 = 98%), 6.9% (95% CI 3.4%–10.3, I^2 = 78%), and 8.4% (95% CI 2.7%–14.0%, I^2 = 89%), respectively (Figure 2). Given that most studies in the meta-analysis were from countries with low TB incidence and that the variability of estimates was higher from countries with intermediate and high TB incidence, the overall (pooled) estimates of ARTI was 4.6% (95% CI 4.1%–5.6%, I^2 = 97%).

TB among HCWs

A median of 34 (IQR 18–108) TB cases occurred among HCWs; each study accounted for a median of

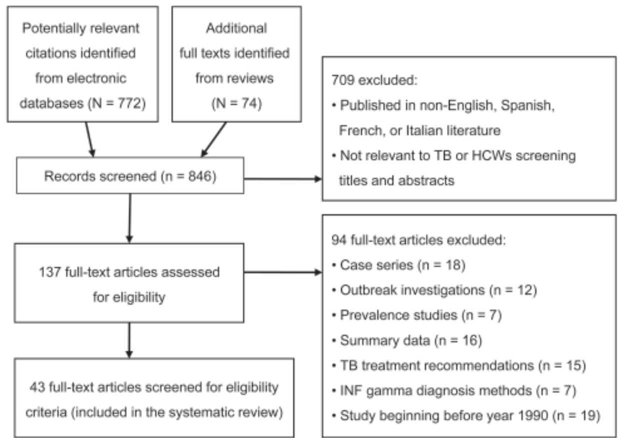

Figure 1. Flow diagram for selection of studies of tuberculosis (TB) in health care workers (HCWs) published during January 2005–July 2010. INF, interferon.

12,689 (IQR 2,979–57,279) person-years. The median estimated annual incidence of TB among HCWs was 67 cases/100,000 persons (IQR 40–142), 91/100,000 persons (IQR 81–723), and 1,180/100,000 persons (IQR 91–3,222) for studies from countries with low, intermediate, and high TB incidence, respectively. The median estimates for TB incidence for the general population adopted as comparisons were 33/100,000 persons (IQR 27–37), 82/100,000 persons (IQR 58–223), and 311/100,000 persons (IQR 168–405), respectively. Median estimated annual TB IRRs were 2.0 (IQR 1.5–4.1), 1.4 (IQR 0.4–8.8), and 5.4 (IQR 1.7–9.1), respectively.

The median differences in TB incidence for countries with low, intermediate, and high TB incidence were 34 cases/100,000 persons (IQR 12–105), 33/100,000 persons (IQR–41 to 641), and 409/100,000 persons (IQR 166–2817), respectively. These findings suggest that 49%, 27%, and 81% of TB cases, respectively, among HCWs were attributable to exposure in health care settings.

For countries with low and intermediate TB incidence, stratified pooled estimates for annual TB IRRs were similar: 2.42 (95% CI 1.20–3.64, I^2 = 85%) and 2.45 (95% CI 1.05–3.84, I^2 = 98%), respectively (Figure 3). The TB IRRs for countries with high TB incidence was higher: 3.68 (95% CI 2.89–4.48, I^2 = 96%%). The overall estimate of annual TB IRR was 2.97 (95% CI 2.43–3.51, I^2= 98%).

For countries with low TB incidence, the proportion of HCWs in the population ranged from 1.7 to 8.2 HCWs per 1,000 residents. For countries with intermediate and high TB incidence, the proportion of HCWs in the population ranged from 3.5 to 12.8 HCWs per 1,000 residents and from 0.6 to 4.8 HCWs per 1,000 residents, respectively.

Using the estimated annual TB IRR and the reported population numbers of HCWs for each country of interest, we estimated the PAF. The median estimated PAF for TB was 0.36% (IQR 0.13–2.24) for studies from countries with low TB incidence, 0.43% (IQR –0.82 to 2.66) for studies from countries with intermediate TB incidence, and 0.38% (IQR 0.14–0.78) for studies from countries with high TB incidence.

For each level of IRR, a nonlinear relationship existed between the PAF and the percentage of HCWs in the population (online Technical Appendix Figure 2). For

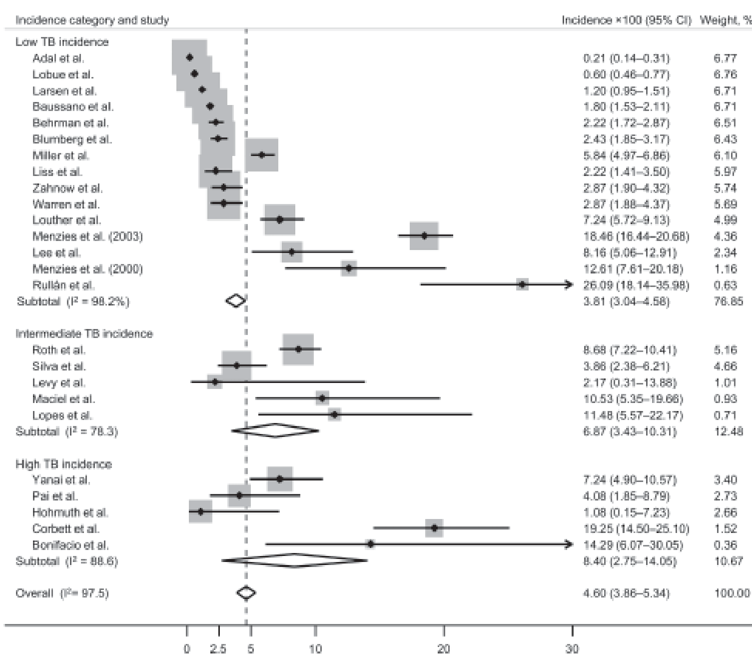

Figure 2. Forrest plot showing study-specific and pooled estimates of the annual incidence of latent *Mycobacteria tuberculosis* infection (LTBI) among health care workers (HCWs), by tuberculosis incidence groups according to the World Health Organization. Studies are sorted by incidence category: low, <50 cases/100,000 population; intermediate, 50–99 cases/100,000 population; high, >100 cases/100,000 population. CI, confidence interval. A complete list of references is provided in the online Technical Appendix (www.cdc.gov/EID/content/16/3/488-Techapp.pdf).

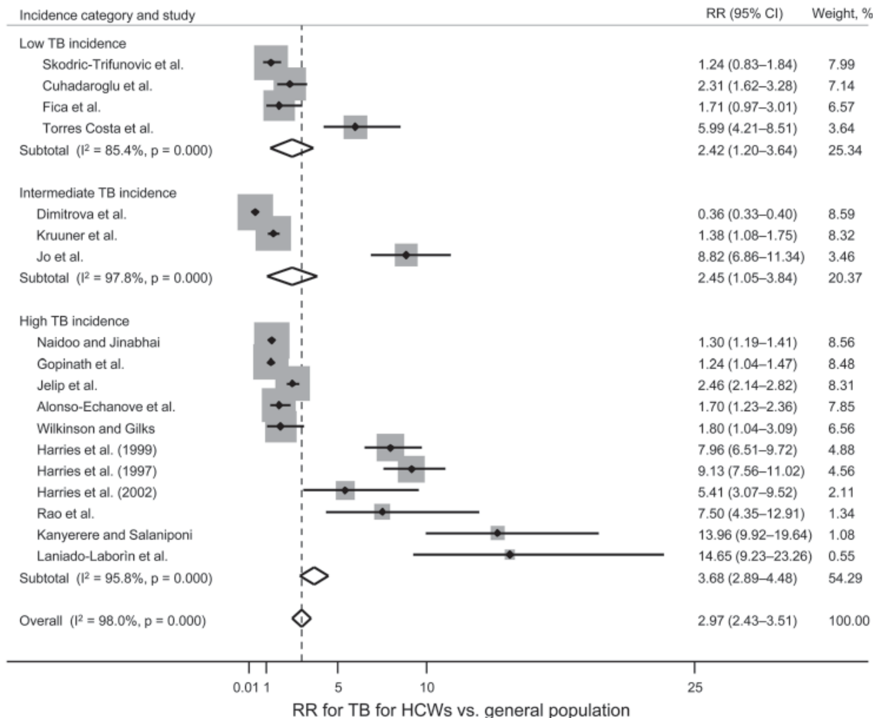

Figure 3. Forrest plot showing the study-specific and pooled estimates of incidence rate ratios (RR) for tuberculosis (TB) among health care workers (HCWs) compared with the corresponding general populations, by TB incidence group according to the World Health Organization. Studies are sorted by incidence category: low, <50 cases/100,000 population; intermediate, 50–99 cases/100,000 population; high, ≥100 cases/100,000 population. CI, confidence interval. A complete list of references is provided in the online Technical Appendix (www.cdc.gov/EID/content/16/3/488-Techapp.pdf).

example, Harries et al. (*14*) and Rao et al. (*15*) reported similar IRRs (7.9 and 7.6, respectively), but because of the different proportion of HCWs working in health care settings in Malawi and India, respectively, the estimated PAF varied widely (0.42% and 1.21%, respectively). On the other hand, Gopinath et al. (*16*) and Rao et al. (*15*) reported from India; thus, the proportion of population working in health care settings was assumed to be the same (1.9 HCWs/1,000 persons, respectively) but the large difference in IRR reported in the 2 studies produced a substantial shift in the estimated PAF (from 0.04% to 1.2%).

The meta-regression model, which accounts for both the effect of country income levels as classified by the World Bank (*13*) and for the HCWs densities as reported by WHO (*8*), has shown that the between-studies variance was significantly reduced (τ^2 decreased from 1.2–0.71). In particular, the IRR for TB among HCWs decreased by ≈10% as the HCW density increased by 1/1,000 persons and by about two thirds in middle-income countries, compared with low-income countries. However, the variability not explained by the information included in the analysis decreased to only 96%. Incorporating the effect of time (i.e., year of publication of the paper) in the analysis did not significantly decrease the heterogeneity between the studies.

Finally, visual inspection of the funnel plot suggested a possible publication bias for the studies reporting TB incidence among HCWs from countries with high TB incidence (online Technical Appendix). A formal testing procedure that used the Begg test, however, failed to confirm such a bias (*17*).

Discussion

The results of our analysis show that the risk for TB among HCWs is consistently higher than the risk among the general population worldwide. This finding confirms that TB is an occupational disease. The introduction of TB transmission control measures, essential to protect HCWs, may decrease TB annual incidence among HCWs by as much as 49%, 27%, and 81% in countries with low, intermediate, and high TB incidence, respectively.

The annual risk for LTBI among HCWs and the TB IRRs, excluding the study by Dimitrova et al. (*18*), increase according to the background TB incidence in the population. The exceptionally low IRR estimated from the study by Dimitrova et al. may be a consequence of our decision to calculate TB IRR by pooling HCWs working in general health services and in TB facilities and considering as a reference TB incidence in the general population of Russia (95/100,000), which was higher than the incidence reported in the Samara Oblast (75/100,000), where the study was conducted. The reported incidence among HCWs working in general health services was 69/100,000, whereas the incidence among staff at the TB services was 741/100,000, which is 10× higher than among the general population.

The high PAF represents the effect of an increase in the HCW population in a specific setting, as may occur in countries undergoing socioeconomic transition, without

the concomitant introduction of TB transmission control measures, and the consequential increased effects of health care settings on the total incidence of TB in the population. In contrast, low PAF estimates might represent the effect of the introduction of TB transmission control measures in health care settings, and the consequential reduced effects of health care settings on total incidence of TB in the population.

Our study suggests that the effect of TB in HCWs (estimated by PAF) on global TB incidence does not exceed 5% and is comparable across countries with different background TB incidence, possibly because the proportion of HCWs and the TB IRRs counterbalance each other. Such estimated PAF figures call for urgent improvement of TB control measures in health care settings, particularly in settings that have a high IRR for TB.

The substantial heterogeneity between studies could be due to differences in methodologic quality, study design, sampling variability, and study populations across studies. In particular, some studies focused specifically on high-risk groups among HCWs, such as laboratory workers (*19–21*) and nurses (*22*). Furthermore, we could not discriminate between settings where infection transmission control measures were or were not implemented. It was equally difficult to classify settings according to their risk for admitting a person with unsuspected TB or according to the characteristics of the population attending the health care facilities, such as homeless persons, drug abusers, or migrants from countries with high TB prevalence.

The analysis restricted to the studies reporting cases among HCWs, regardless of their specific professional role, did not show any significant variation of the IRR estimates. Whether the HCWs investigated were representative of the national population of HCWs for each reported study was impossible to know; thus, IRR estimates resulting from the comparison of their risk for TB with the risk for the general population could be nonrepresentative of the national estimates. The density of HCWs and the level of income of each country, considered simultaneously in the analysis, accounted for some between-study variance. The IRR for TB decreased with the proportion of the population working in health care settings and in middle-income countries compared with high-income countries. Unfortunately, however, much of the heterogeneity still remained unexplained.

The method adopted to estimate the PAF typically measures the effects on a population of risk factors for noncommunicable diseases (*11*), ignoring the specific component of TB transmission dynamics between local community and health care settings (*23*). Thus, the PAF estimates presented here should be considered a lower limit for the real effects of controlling TB transmission in health care settings, accounting for the remaining 2 main components acting on such an effect: the proportion of the population working in health care settings and the role of transmission control measures. Furthermore, PAF estimates do not account for patient-to-patient transmission of TB in health care facilities. Patient-to-patient transmission could constitute a major pathway of TB transmission, especially in settings of high HIV prevalence. Control of TB transmission in such a group could appreciably reduce the overall incidence of TB in the general population. However, data on TB transmission among patients are scanty and difficult to analyze in the context of this systematic review.

Although the cost-effectiveness assessment of the introduction of TB transmission control measures, encapsulated in the WHO Stop TB Strategy (*24*), is beyond the scope of this article, such a potential reduction of TB incidence among HCWs would make attractive a range of infection transmission control strategies. Managerial activities, administrative controls, adoption of N95 respirators, and engineering controls all represent potentially effective measures. The implementation of a sound TB infection control package based on the facility assessment, is hampered by constraints in poor resource settings; nevertheless, administrative controls alone have been proven effective in decreasing the risk for TB among HCWs (*25–27*).

In conclusion, our findings show that HCWs are at higher than average risk for infection with *Mycobacterium tuberculosis* and of developing TB disease. For this reason, sound TB infection control measures, including early diagnosis and prompt treatment of infectious cases, should be prioritized. Drug resistance emphasizes the urgency for implementing such measures, which would also benefit reduction of patient-to-patient transmission in health care facilities.

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Laboratory-based Surveillance of Extensively Drug-Resistant Tuberculosis, China

Yunfeng Deng, Yan Wang, Junling Wang, Hui Jing, Chunbao Yu, Haiying Wang, Zhimin Liu, Edward A. Graviss, and Xin Ma

To estimate the prevalence of extensively drug-resistant tuberculosis (XDR TB) in China, we retrospectively analyzed drug-resistance profiles of 989 clinical *Mycobacterium tuberculosis* isolates. We found 319 (32.3%) isolates resistant to ≥1 first-line drugs; 107 (10.8%) isolates were multidrug resistant, of which 20 (18.7%) were XDR. XDR TB is of major concern in China.

Extensively drug-resistant (XDR) tuberculosis (TB), a severe form of TB disease, is defined as TB that is resistant to at least rifampin and isoniazid (multidrug resistant [MDR]), as well as to any member of the quinolone family and at least 1 second-line anti-TB injectable drug: kanamycin, capreomycin, or amikacin (*1*,*2*). According to the World Health Organization (WHO), XDR TB has been reported in 57 countries and is a major concern for global health (*2*,*3*). The WHO Global Task Force on XDR TB has recommended laboratory-based surveillance to better understand the prevalence of XDR TB in developing countries (*4*). However, surveillance data on XDR TB from People's Republic of China remain scant. Shandong Province is the second largest province in China, with a population of 94 million. Shandong Provincial Chest Hospital (SPCH) is the only provincial-level hospital specializing in TB clinical service and control. In collaboration with the SPCH TB reference laboratory, we retrospectively analyzed the drug-resistance profiles of a group of clinical *Mycobacterium tuberculosis* isolates to estimate the prevalence of XDR TB in China.

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The Study

During November 2004–April 2007, a total of 989 clinical *M. tuberculosis* isolates were cultured and examined by first- and second-line anti-TB drug susceptibility test (DST) at the SPCH TB reference laboratory. These isolates were collected from 989 inpatients (mean age ± SD 40.1 ± 18.9 years; range 0.3–88 years; 65.5% male) at the SPCH,; these patients represented 860 (87.0%) new and 129 (13.0%) retreatment TB cases. The DST was performed according to WHO-recommended standard procedures, and quality control was conducted by interlaboratory confirmation tests with WHO-recognized reference laboratories in South Korea and Hong Kong Special Administrative Region, China (*5*,*6*). The DST panel included 4 first-line anti-TB drugs: isoniazid, rifampin, streptomycin, and ethambutol, and 5 second-line drugs: para-aminosalicylic acid, ciprofloxacin, levofloxacin, amikacin, and capreomycin. Kanamycin was not included in the DST panel because it is rarely used to treat TB disease in this study population because of side effects. Because levofloxacin and ciprofloxacin are fluoroquinolones with full cross-resistance, they were considered as the same family of anti-TB drugs and represented by fluoroquinolones in our analysis.

Among the 989 *M. tuberculosis* isolates, the overall proportion of first-line drug resistance (at least 1 drug) was 32.3% (319/989). streptomycin had the highest rate of resistance (24.1%), followed by isoniazid (18.9%), rifampin (16.1%), and ethambutol (4.7%). A total of 107 (10.8%) isolates were resistant to at least isoniazid and rifampin (MDR). Thirty-one (3.1%) isolates were resistant to all first-line drugs (Table 1). Eighty-three MDR isolates (77.6%) were identified from new TB case-patients. The overall rate of second-line drug resistance was 19.1% (189/989). Fluoroquinolones had the highest rate of resistance (16.4%), followed by capreomycin (5.7%), para-aminosalicylic acid (3.7%), and amikacin (3.2%). A total of 27 (2.7%) isolates were resistant to ≥3 second-line drugs.

Among the 107 MDR isolates, 60.7% (65/107) were resistant to at least 1 second-line drug, and 53.3% (57/107) were resistant to fluoroquinolones. A total of 20 (18.7%) MDR isolates met the definition of XDR TB (resistant to any fluoroquinolones and at least 1 injectable drug) (Table 2). Among 20 XDR isolates, 11 were resistant to 4 first-line anti-TB drugs and 10 to >6 first- and second-line anti-TB drugs. The 20 XDR isolates were cultured from the sputum specimens of 20 patients with pulmonary TB (mean age ± SD 47.0 ± 15.8 years; range 18–68 years; 11 male). Seventeen patients with XDR TB were receiving retreatment and had 4–30-year histories of chronic TB and had been previously treated with second-line anti-TB drugs. Three patients with XDR TB had new cases without prior anti-TB treatment. Contact investigations did not identify epidemiologic links among these patients with XDR TB.

Table 1. First- and second-line drug resistance of 989 clinical *Mycobacterium tuberculosis* isolates, People's Republic of China, November 2004–April 2007*

Drugs*	No. isolates	Rate, %
Overall first-line drug resistance	319	32.3
INH	187 (44)	18.9 (4.4)
RFP	159 (16)	16.1 (1.6)
EMB	46 (0)	4.7 (0)
SM	238 (78)	24.1 (7.9)
MDR, overall	107	10.8
INH + RFP	16	1.6
INH + RFP + EMB	2	0.2
INH + RFP + SM	58	5.9
INH + RFP + EMB + SM	31	3.1
Overall second-line drug resistance	189	19.1
FQ	162 (103)	16.4 (10.4)
AMK	32 (0)	3.2 (0)
CPM	56 (14)	5.7 (1.4)
PAS	37 (6)	3.7 (0.6)
Second-line drug polyresistance	66	6.6
FQ + AMK	8	0.8
FQ + AMK + CPM	9	0.9
FQ + AMK + PAS	4	0.4
FQ + AMK + CPM + PAS	6	0.6
FQ + CPM	15	1.5
FQ + PAS	10	1.0
FQ + CPM + PAS	7	0.7
AMK + CPM	3	0.3
AMK + PAS	1	0.1
AMK + CPM + PAS	1	0.1
CPM + PAS	2	0.2

*Numbers and rates of mono–first- and -second–line drug-resistant strains shown in parentheses. INH, isoniazid; RFP, rifampin;, EMB, ethambutol; SM, streptomycin; MDR, multidrug-resistant; FQ, fluoroquinolines (specifically ciprofloxacin and levofloxacin); AMK, amikacin; CPM, capreomycin; PAS, para-aminosalicylic acid.

Conclusions

The Global Project on Anti-tuberculosis Drug Resistance Surveillance (2002–2007, 37 countries) has reported that XDR TB prevalence among MDR TB cases ranged from 6.6% to 23.7% worldwide (*1*). The most recent surveillance data from Beijing and Shanghai, China, showed that the XDR TB cases accounted for ≈6.3% of MDR TB cases in both cities (*7,8*). By analyzing first- and second-line drug resistance profiles of 989 clinical *M. tuberculosis* isolates in a clinical laboratory of Shandong Province, we showed that 18.7% of MDR strains met the definition for XDR, which is relatively higher than the previous surveillance data in China (*7,8*). Several issues might explain this deviation. First, the data from Shanghai were obtained through a population-based Shanghai Center for Disease Control surveillance mechanism that included general hospitals, TB clinics, and community health centers, whereas our data were obtained through a TB hospital–based surveillance study with a relatively higher proportion of previously treated patients (chronic or refractory TB cases with prior anti-TB treatment) than in the Shanghai study.

Table 2. Second-line drug resistance of 107 MDR *Mycobacterium tuberculosis* isolates, People's Republic of China, November 2004–April 2007*

Drugs	No. isolates	Rate, %
Overall second-line drug resistance	65	60.7
FQ	57	53.3
AMK	19	17.8
CPM	24	22.4
PAS	18	16.8
XDR, total	20	18.7
FQ + AMK	4	3.7
FQ + CPM	4	3.7
FQ + AMK + CPM	5	4.7
FQ + AMK + PAS	2	1.9
FQ + CPM + PAS	3	2.8
FQ + AMK + CPM + PAS	2	1.9

*MDR, multidrug-resistant; FQ, fluoroquinolines (specifically ciprofloxacin and levofloxacin); AMK, amikacin; CPM, capreomycin; PAS, para-aminosalicylic acid; XDR, extensively drug-resistant.

Therefore, the data from our study may overestimate the prevalence of XDR and MDR TB in Shandong Province. Second, the data from the Beijing study also were obtained through a TB hospital–based surveillance study. However, the DST panel did not include capreomycin, which may have led to an underestimation of the XDR TB prevalence among inpatients of this TB hospital.

The susceptibility testing of second-line anti-TB drugs has not been standardized (*9,10*). Because second-line anti-TB drugs are being prescribed more frequently in current clinical practice, quality assurance and clinical correlation of second-line DST are urgently needed to provide reliable evidence for clinical management of XDR TB (*9,10*).

The current standard care of TB patients in China (National Tuberculosis Program) does not include the first- and second-line anti-TB DST because of its prohibitive cost. In the current study, 15.0% of XDR and 77.6% of MDR isolates were obtained from persons for whom TB was newly diagnosed and who had received no prior anti-TB treatment (i.e., had primary drug resistance). The surveillance data from Shanghai have also shown that more than half of XDR and MDR TB cases occurred in patients for whom TB was newly diagnosed (*8*). These results clearly indicate that the transmission of drug-resistant TB among Chinese populations is extensive and widespread, which highlights a need for TB control policy reform in China to face this emerging challenge.

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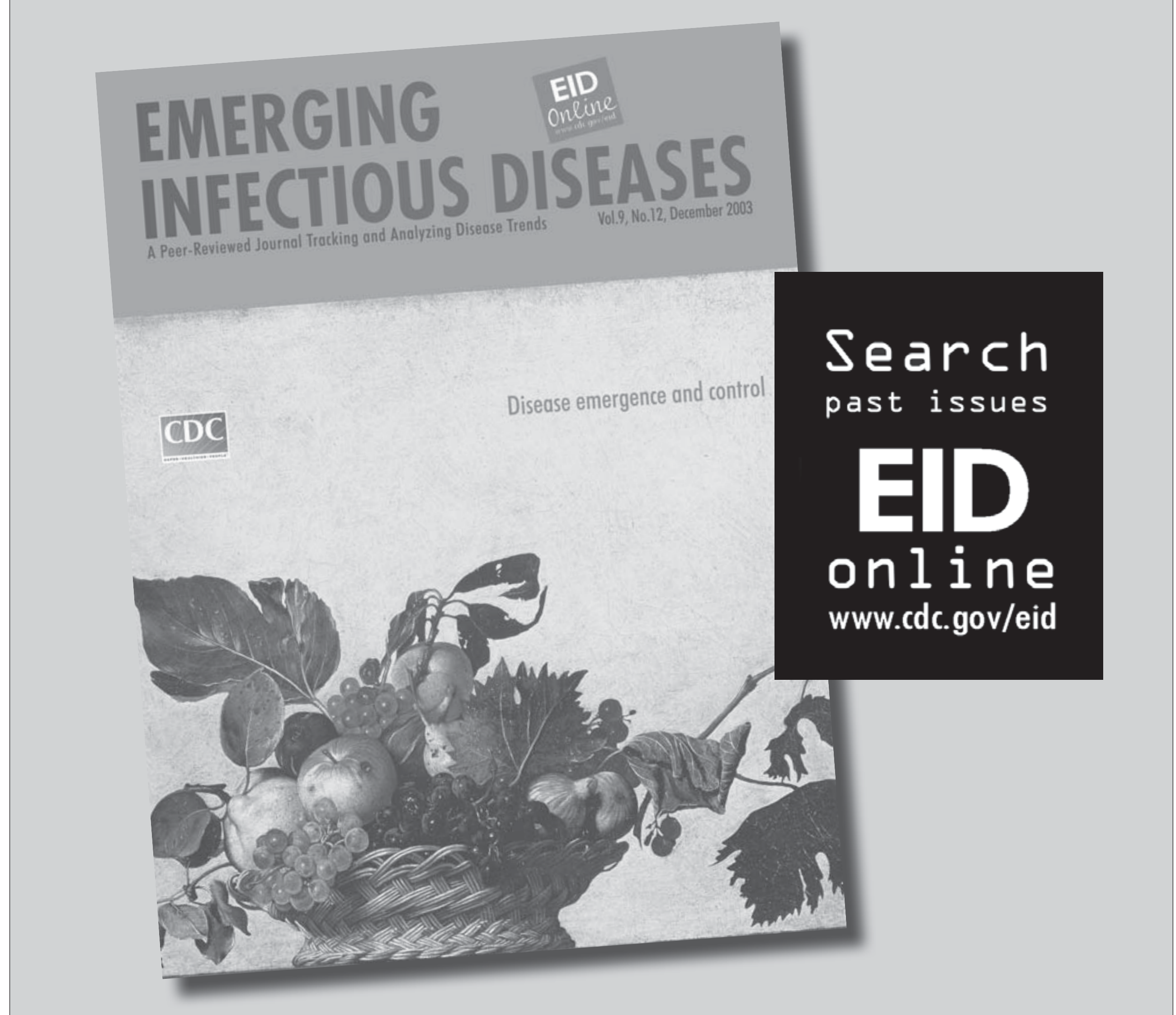

Multidrug-Resistant Genotypes of *Plasmodium falciparum*, Myanmar

Zhaoqing Yang,[1] Chaoqun Li,[1] Miao Miao, Zaixing Zhang, Xiaodong Sun, Hao Meng, Jie Li, Qi Fan, and Liwang Cui

We performed a molecular epidemiologic survey of mutations associated with drug-resistance genes in *Plasmodium falciparum* in northeastern Myanmar. In this region, 3 highly mutated drug-resistance haplotypes and 1 associated with decreased quinine susceptibility were prevalent, which suggests that parasites may be resistant to multiple commonly used antimalarial drugs.

Malaria is a major impediment to socioeconomic development in the Greater Mekong Subregion (GMS) of Southeast Asia (*1*). Malaria distribution in the GMS is extremely uneven, with areas of high endemicity in some countries and along international borders. In Myanmar, malaria is particularly problematic; more than half of malaria cases and approximately three fourths of malaria-related deaths in the GMS during 2007 occurred in Myanmar. The GMS has been the breeding ground of multidrug-resistant *Plasmodium falciparum*, and resistance to chloroquine and antifolates arose there and spread to Africa (*2*,*3*). In particular, recent detection of reduced artemisinin susceptibility at the Thailand–Cambodia border is a major concern (*4*). As a result, drug resistance has been monitored extensively in this region. In contrast, information about resistance to antimalarial drugs in Myanmar is exceptionally scarce. Accordingly, as our initial step toward a comprehensive antimalarial drug study in Myanmar, we performed a molecular survey of drug resistance in the northeastern region of this country.

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The Study

We screened by microscopy 4,980 patients with febrile illness who sought care at a malaria clinic in Kachin State, northeastern Myanmar, during 2007–2009; a total of 27.9% had malaria infections. *P. falciparum, P. vivax,* and mixed species infections accounted for 56.7%, 41.1%, and 2.2% of malaria cases, respectively. Finger-prick blood samples were obtained from 260 patients with uncomplicated *P. falciparum* infection who had not used antimalarial drugs during the previous 2 weeks. Parasite DNA was extracted from filter papers and genotyped at 3 polymorphic genes, which detected 54.6% of samples containing mixed-strain infections (*5*). Of the 118 samples with monoclonal infection, 117 samples were successfully genotyped by PCR and sequencing at 5 known and putative drug-resistance genes (online Technical Appendix Table, www.cdc.gov/eid/content/17/3/498-Techapp.pdf), some of which have been widely used for resistance surveillance and as predictors of clinical efficacy of antimalarial drugs.

Sequencing of 2 fragments in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene covering single nucleotide polymorphisms (SNPs) at codons 72–76 and 220, respectively (*6*), showed that the major chloroquine resistance determinant K76T mutation has reached fixation in the parasite population (Figure 1). All parasites had sequence CVIET at positions 72–76, compared with the wild-type sequence SVMNK. In addition, the A220S mutation associated with chloroquine resistance was predominant (99.1%).

Sequencing of 2 *P. falciparum* multidrug resistance 1 (*pfmdr1*) fragments as described (*5*) detected only the N86Y, Y184F, and N1042D mutations making up 5 haplotypes (Figure 2). The overall haplotype prevalence differed significantly among the years ($p<0.001$, $\chi^2 = 31.39$, df = 8).The prevalence of wild-type haplotype was 59.0%. Among the 3 mutant codons, only Y184F reached a relatively high frequency (35.9%). One sample contained the double mutations 184F/1042D. Analysis of *pfmdr1* copy number from monoclonal infections by real-time PCR with 3D7 and Dd2 strains as negative and positive controls, respectively (*6*), detected no *pfmdr1* amplification.

Sequencing of the dihydrofolate reductase (*pfdhfr*) and dihydropoteroate synthase (*pfdhps*) genes (online Technical Appendix Table) detected 4 SNPs in *pfdhfr* associated with pyrimethamine resistance (N51I, C59R, S108N, and I164L) and 4 SNPs in *pfdhps* associated with sulfadoxine resistance (S436A, A437G, K540E/N, and A581G) (Table 1). The overall haplotype prevalence of the 2 genes differed significantly between the years ($p<0.0001$, $\chi^2 = 76.49$, df = 28). Of the 5 *pfdhfr* haplotypes, wild-type NCSI was observed only in 1 sample in 2007; the remaining samples

[1]These authors contributed equally to this article.

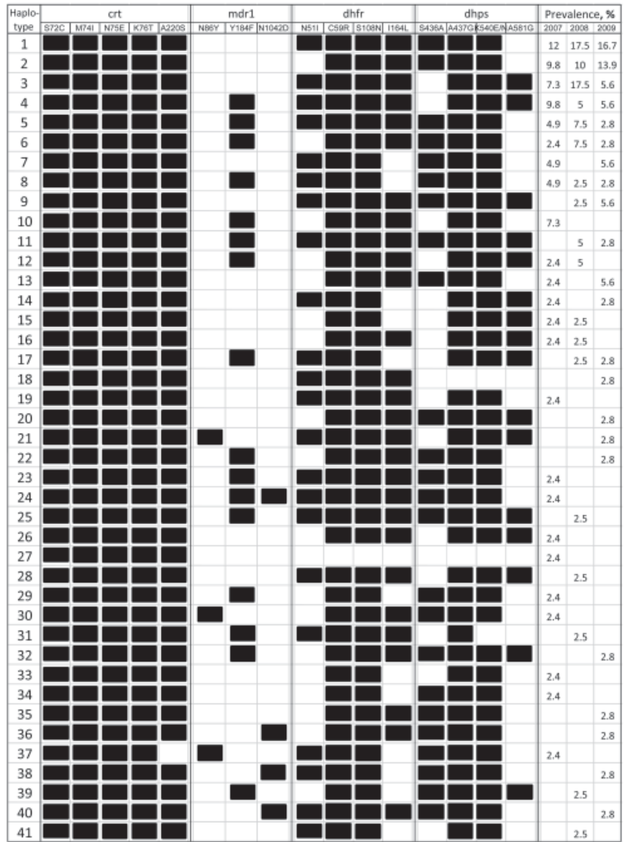

Figure 1. Multilocus genotypes in *Plasmodium falciparum* isolates, Kachin State, northeastern Myanmar, 2007–2009. A total of 41 haplotypes were identified from 117 parasite isolates. Wild-type and mutated amino acids are shown in white and black, respectively. Prevalence (%) of each multilocus genotype in each year is indicated in the right columns.

contained at least double mutations 59R/108N. Two triple-mutation haplotypes (N**RNL** and **IRN**I, mutations in **boldface**) were detected with N**RNL** being more frequent than **IRN**I in each year. Overall, quadruple mutations (**IRNL**) were found in >50% of the samples. In addition, frequency of triple and quadruple mutations increased gradually from 2007 to 2009. We found all 5 haplotypes in 2007 but only triple and quadruple mutations in 2009. In *pfdhps*, 10 haplotypes were found, and 437G and 540E/N mutations were highly prevalent: 98.3 and 96.6%, respectively (Table 1). Similarly, the wild-type *pfdhps* haplotype SGKA was found in only 2 samples. **AGE**A was the most common haplotype in each year and reached an overall frequency of 48.7%. Quadruple mutations (**AGEG**) were found only in 2008 and 2009.

Molecular analysis of drug-resistance markers in monoclonal infections enabled us to obtain multilocus genotypes of the parasites. Genotyping each of the 117 parasite isolates at 16 drug resistance–related codons in the *pfcrt*, *pfmdr1*, *pfdhfr*, and *pfdhps* genes showed 41 haplotypes (Figure 1). Among these haplotypes, parasites containing ≥10 mutated codons accounted for 93.2% of the samples.

Polymorphisms in the minisatellite ms4760 of *P. falciparum* Na^+/H^+ exchanger (*pfnhe1*) are associated with quinine sensitivity (*5,7–10*). Sequencing of the *pfnhe1* fragment containing the ms4760 minisatellite from 79 monoclonal infections showed 10 haplotypes, with haplotype 7 the most predominant (54.4%) (Table 2). More than 64% of samples tested contained ≥3 copies of the DNNND repeat (Rep1); 76% contained 1 copy of the NHNDNHNNDDD repeat (Rep2). Accordingly, >60% of parasite isolates had a Rep1:Rep2 ratio of ≥3:1.

Conclusions

In Myanmar, high-level resistance to chloroquine and pyrimethamine–sulfadoxine was reported more than a decade ago (*11–13*). Our molecular survey showed that the major chloroquine resistance allele CVIET has reached fixation, and triple and quadruple mutations in *pfdhfr* and *pfdhps* were highly prevalent in this region. These findings strongly suggest that a large proportion of parasites might show clinical resistance to chloroquine and antifolate drugs. Although chloroquine has been withdrawn from treating *P. falciparum* malaria for decades in some regions,

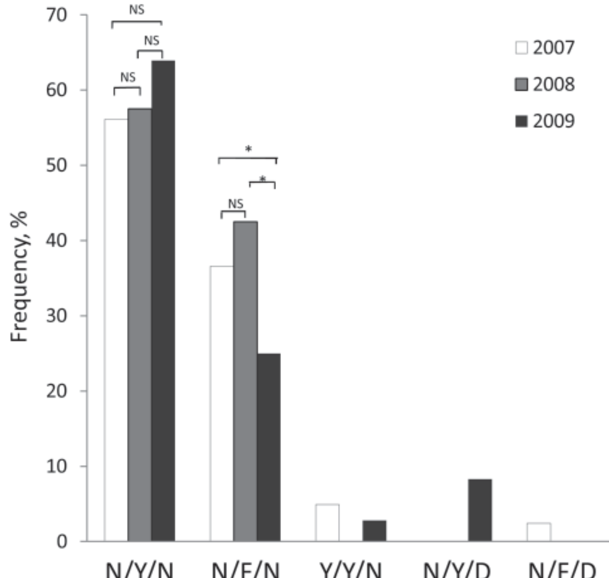

Figure 2. Annual prevalence of *Plasmodium falciparum* dihydrofolate reductase haplotypes among clinical samples collected from Kachin State, northeast Myanmar, 2007–2009. The x-axis shows the 5 haplotypes (the amino acids at positions 86, 184, and 1042 with mutated amino acids in **boldface**). The χ^2 test was performed to compare prevalence of 2 major haplotypes between years. For each haplotype, NS denotes no significant difference (p>0.05) between years; asterisk (*) denotes significant difference (p<0.05) between years.

Table 1. Prevalence of point mutation haplotypes in *pfdhfr* and *pfdhps* in clinical samples from Kachin State, northeast Myanmar, 2007–2009*

Gene	Haplotype	Codon†	Haplotype prevalence,‡ %		
			2007, n = 41	2008, n = 40	2009, n = 36
Pfdhfr (51, 59, 108, 164)	Wild-type	NCSI	2.4	–	–
	Double mutations	N**RN**I	9.8	5.0	–
	Triple mutations	N**RNL**	31.7[a]	25.0[b]	36.1[a]
		IRNI	14.6[a]	7.5[b]	16.7[a]
	Quadruple mutations	**IRNL**	41.5[a]	62.5[b]	47.2[a]
Pfdhps (436, 437, 540, 581)	Wild-type	SAKA	2.4	–	2.8
	Single mutation	S**G**KA	–	2.5	2.8
	Double mutations	S**GE**A	9.8	2.5	–
		S**GN**A	2.4	–	–
	Triple mutations	S**GEG**	26.8[a]	35.0[b]	19.4[c]
		S**GNG**	2.4	2.5	–
		AGEA	48.8[a]	45.0[a]	52.8[a]
		AGNA	7.3	–	8.3
	Quadruple mutations	**AGEG**	–	10.0	11.1
		AGNG	–	2.5	2.8

**Pfdhfr, Plasmodium falciparum* dihydrofolate reductase; *Pfdhps, P. falciparum* dihydropoteroate synthase; –, no such haplotype detected.
†Codons in **boldface** indicate mutated amino acids.
‡χ^2 test was performed with all data points of the 2 genes, which showed that overall haplotype prevalence differed significantly between the years (χ^2 test, p<0.0001, χ^2 = 76.49, df = 28). Major *dhfr* and *dhps* haplotypes were individually compared. For each haplotype (in the same row), the labeling by different letters denotes significant difference between the years (χ^2 test, p<0.05).

the *pfcrt* resistance alleles showed no sign of abating (*6*). Furthermore, despite adoption of artemisinin combination therapy in 2002, the frequency of highly mutated *pfdhfr* and *pfdhps* haplotypes appeared to have increased during this study, which suggested that artemisinin combination therapy might not have retarded the spread of antifolate-resistant parasites. This situation differs from that in the western Myanmar border area but is similar to that in Thailand and Cambodia, where highly mutated *pfdhfr* and *pfdhps* genotypes also were common (*14*,*15*).

Mutations in *pfmdr1* are associated with resistance to several antimalarial drugs including chloroquine, mefloquine, and quinine and increased *pfmdr1* copy number is responsible for mefloquine resistance. We found that ≈60% of the parasites contained the wild-type *pfmdr1* allele, similar to some parasites from the western Myanmar border area (*15*). No *pfmdr1* amplification was detected, suggesting that parasites from this region might be mefloquine sensitive, consistent with the fact that mefloquine has not been deployed here. In contrast, in vitro mefloquine resistance was observed in southeast Myanmar bordering Thailand (*13*), possibly because of the extensive use of mefloquine in Thailand for the past 2 decades.

Table 2. Prevalence of the *pfnhe1* minisatellite ms4760 haplotypes in the clinical samples from Kachin State, northeast Myanmar, 2007–2009*

Haplotype†	No. rep 1	No. rep 2	Haplotype frequency, %
1	2	2	12.7
3	1	2	5.1
5	4	1	3.8
6	2	1	13.9
7	3	1	54.4
9	3	2	3.8
14	3	1	1.3
18	2	2	2.5
34	4	1	1.3
35	1	1	1.3

**Pfnhe1*, *Plasmodium falciparum* Na^+/H^+ exchanger.
†Numbers refer to the haplotype list of Meng et al. (*5*).

Although the validity of *pfnhe1* minisatellite polymorphism for predicting quinine resistance remains uncertain and may depend on the parasites' origins (*7*–*9*), we detected significant association of decreased quinine susceptibility with increased DNNND repeat copies (*5*). We have provided further evidence on the high prevalence of parasites with increased DNNND repeats in *pfnhe1*, which suggests that some parasite strains might show reduced sensitivity to quinine.

Overall, our molecular survey of antimalarial drug resistance in *P. falciparum* showed high frequency of multidrug-resistant haplotypes in northeastern Myanmar. Moreover, parasites in this region had unique multilocus genotypes that differed markedly from those in other areas of the GMS. These findings suggest that coordinated efforts are necessary to thwart the spread of resistant strains across larger geographic regions. Our molecular study showed only the genotypes of the drug resistance genes; further in vitro and in vivo studies are required to corroborate these findings.

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Livestock-associated Methicillin-Resistant *Staphylococcus aureus* in Humans, Europe

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To estimate the proportion of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from humans that were sequence type (ST) 398, we surveyed 24 laboratories in 17 countries in Europe in 2007. Livestock-associated MRSA ST398 accounted for only a small proportion of MRSA isolates from humans; most were from the Netherlands, Belgium, Denmark, and Austria.

Livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA) was first associated with human disease in 2003, when a MRSA clone associated with a reservoir in pigs and cattle was isolated from a human. This clone was not typable by pulsed-field gel electrophoresis with *Sma*I macrorestriction digestion and belonged to multilocus sequence type (ST) 398 (*1*). Since then, rates of MRSA ST398 carriage have been high (25%–35%) for persons in the Netherlands who have frequent contact with pigs and veal calves, but associated illness is rare (*2*). However, in Europe, Asia, and the United States, invasive infections and a hospital outbreak of MRSA ST398 have been reported (*3*). We estimated the proportion of MRSA isolates from humans in Europe in 2007 that were ST398.

The Study

Questionnaires were mailed to 43 laboratories in 23 European countries, selected on the basis of expertise and publications about MRSA. Questions asked for level of laboratory and typing methods used, number of MRSA isolates identified in 2007, number of these isolates that were typed, and number of typed isolates that were MRSA ST398. MRSA isolates were considered to be ST398 if they 1) belonged to multilocus ST398, 2) were *spa* types t011, t034, t108, t567, t571, t588, t753, t898, t899, t1184, t1254, t1255, t1451, t1456, t1457, t2123, t2330, t2383, t2582, or t3013 (*4*; National Institute for Public Health and the Environment, unpub. data); or 3) were not typable by pulsed-field gel electrophoresis with *Sma*I macrorestriction digestion. Laboratories were asked to report data on clinical isolates only (as opposed to screening isolates) and to provide the distribution by body site.

For each laboratory, the proportion of MRSA ST398 among all typed MRSA isolates from humans and the 95% Wilson confidence interval (CI) were calculated. For laboratories that typed all MRSA isolates, χ^2 testing compared proportions of isolates from various body sites for MRSA ST398 isolates and for other MRSA isolates.

For each country, we compared the proportions of MRSA ST398 among human MRSA isolates with number of pigs per km^2, number of cattle <1 year of age (a surrogate for veal calves) per km^2, and 2 indices multiplying these animal densities with human population densities.

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Criteria were that a laboratory had to report ≥100 MRSA isolates and type >25% of those isolates, thus leaving 14 national or regional laboratories from 12 countries. For Austria, data from 2 laboratories were pooled because these laboratories did not report duplicate isolates. For Denmark, only data on MRSA clinical isolates were used. Data for 2007 on midyear human population, pig production, and production of cattle <1 year of age were obtained from Eurostat (http://epp.eurostat.ec.europa.eu) except for pig production in Switzerland (Swiss Statistics, www.bfs.admin.ch) and Turkey (Turkstat, www.turkstat.gov.tr). Land area was obtained from The World Factbook (www.cia.gov/library/publications/the-world-factbook). For Germany, 1 region with high pig density was considered separately from the rest of the country. Data for this region (Eurostat regional Nomenclature of Territorial Units for Statistics code DEA3, corresponding to laboratory 8) were obtained from Eurostat, the Chamber for Agriculture Nordrhein-Westfalen: "Zahlen zur Landwirtschaft 2008" (www.landwirtschaftskammer.de/wir/pdf/zahlen-landwirtschaft-2008.pdf), and the statistical office of Nordrhein-Westfalen in Germany.

Questionnaires were received from 24 laboratories (response rate 56%) in 17 countries. Data from Malta and Slovenia and from 1 laboratory in Italy were not analyzed because these laboratories did not type MRSA isolates. Among the remaining 15 countries, 8 countries reported a combined total of 8,262 MRSA isolates with typing results, of which 142 (1.7%, 95% CI 1.5–2.0%) were MRSA ST398 (Table 1). The proportions of MRSA ST398 per country were 0–11.9%; the countries with the highest proportion were the Netherlands (11.9%), Belgium (4.7%), Denmark (1.6%), and Austria (1.4%, pooled data). The proportion of isolates from blood was significantly lower for MRSA ST398 than for other MRSA clinical isolates. No difference was observed for other body sites (Table 2).

The proportion of MRSA ST398 among human MRSA isolates correlated with pig density (Spearman $\rho = 0.79$, $p = 0.001$) and with the index combining this density with human population density (Spearman $\rho = 0.76$, $p = 0.002$). The proportion of MRSA ST398 among human MRSA isolates also correlated, although less significantly, with the density of cattle <1 year of age (Spearman $\rho = 0.61$, $p = 0.05$) and with the index combining this density with human population density (Spearman $\rho = 0.74$, $p = 0.01$).

Conclusions

Livestock-associated MRSA ST398 was reported from 8 of 15 European countries. Except for 4 countries and 1 region in Germany, the proportion of MRSA ST398 among MRSA isolates from humans was <2%, suggesting that in 2007 this livestock-associated clone contributed to only a small fraction of all MRSA in humans. A recent study of laboratories in 26 European countries during September 2006–February 2007 found no MRSA ST398 among *S. aureus* isolates from persons with invasive infections (*5*).

Table 1. Characteristics of laboratories that reported MRSA and livestock-associated MRSA ST398 isolates from human samples, Europe, 2007*

Laboratory no.	Country	Type of laboratory	Source of MRSA isolates	No. MRSA isolates received	No. MRSA isolates typed	MRSA ST398 isolates	
						No. (%)	95% CI
1	Austria	National ref	All	523	523	0	0–0.7
2	Austria	National ref	All	586	586	16 (2.7)	1.7–4.4
3	Belgium	National ref	All	329	149	7 (4.7)	2.3–9.4
4	Czech Republic	National ref	Blood	37	10	0	0–27.8
5	Denmark	National ref	All	659	659	14 (2.1)	1.3–3.5
	Denmark	National ref	Clinical	370	370	6 (1.6)	0.7–3.5
6	Finland	National ref	All	1,323	1,323	1 (0.1)	0–0.4
7	Germany	National ref	Clinical	1,293	1,293	9 (0.7)	0.4–1.3
8	Germany	Regional ref	Clinical	866	866	37 (4.3)	3.1–5.8
9	Greece	National ref	Clinical	336	336	0	0–1.1
10	Hungary	National ref	All	365	63	0†	NA
11	Iceland	National ref	Clinical	21	21	0	0–15.5
12	Italy	National ref	Clinical	108	108	1 (0.9)	0.2–5.1
13	Ireland	National ref	Clinical	832	696	0	0–0.5
14	The Netherlands	National ref	Clinical	478	478	57 (11.9)	9.3–15.1
15	The Netherlands	Local	Clinical	12	12	3 (25.0)	8.9–53.2
16	Sweden	National ref	All	1,127	1,127	8 (0.7)	0.4–1.4
17	Switzerland	Local	Clinical	587	65	0†	NA
18	Switzerland	Regional ref	All	182	182	0	0–2.1
19	Switzerland	Regional ref	Clinical	64	64	0	0–5.7
20	Switzerland	Local	All	80	78	0	0–4.7
21	Turkey	Local	Clinical	198	60	0	0–6.0

*MRSA, methicillin-resistant *Staphylococcus aureus*; ST398, sequence type 398; CI, confidence interval; ref, reference laboratory; NA, not applicable.
†Not reported because laboratory typed <25% of MRSA isolates.

Table 2. Distribution of typed MRSA ST398 and other MRSA clinical isolates, by body site, 7 European countries, 2007*

Sample source	No. (%) typed clinical isolates		p value†
	MRSA ST398, n = 113	Other MRSA, n = 3,435	
Blood	2 (1.8)	343 (10.0)	**0.004**
Respiratory tract	20 (17.7)	451 (13.1)	0.16
Skin and wound	76 (67.3)	2,312 (67.3)	0.99
Urinary tract	6 (5.3)	173 (5.0)	0.90
Other	9 (8.0)	156 (4.5)	0.09

*Only data from 9 national or regional laboratories in the 7 countries that reported clinical isolates and typed all these isolates were included. **Boldface** indicates statistical significance (p<0.05). MRSA, methicillin-resistant *Staphylococcus aureus*; ST398, sequence type 398.
†χ^2 test.

MRSA ST398 has been isolated from human samples from Austria (*5*), the Netherlands (*2*), Belgium (*6*), Italy (*7*), Spain (*8*), Germany (*9*), Portugal (*10*), Denmark (*11*), the Czech Republic (*12*), Sweden (*13*), and France (*14*). This study demonstrated MRSA ST398 in human samples in Switzerland and Finland. Although few data have been published on the proportion of MRSA ST398 in Europe, Springer et al. (*15*) reported that during 2006 through mid-2008, among 1,043 human MRSA isolates in Austria, 21 (2.0%) were MRSA ST398, which is similar to the proportion (1.4%, pooled data) found in our study.

Among isolates from blood, a significantly lower proportion were MRSA ST398 than other MRSA. This finding suggests that MRSA ST398 is associated with less severe disease, as indicated (*5*).

The proportion of MRSA ST398 among human MRSA isolates in European countries correlated with pig and veal calf densities and with an index combining pig or veal calf density and human population density. In addition to the well-documented risk factor of occupational exposure to pigs and veal calves, proximity of humans to pigs and veal calves may contribute to transmission of MRSA ST398 from animals to humans. However, the fact that farms are not equally distributed throughout a country may explain the higher proportion of MRSA ST398 among MRSA isolates from humans in certain European countries and regions.

Use of readily available data bears some limitations. Laboratories were not randomly selected, which could result in selection bias. However, bias was limited because most laboratories were national reference laboratories that routinely collect MRSA isolates countrywide. Also, countries may have active national or local screening policies, may select which isolates to type, and may use typing techniques that are not always fully comparable. To minimize these variations, when possible we reported on data from clinical isolates only and excluded data from laboratories that reported few isolates and did not type most MRSA isolates. We also provided a list of MRSA *spa* types that at the time of the study had been identified as corresponding to MRSA ST398. Other *spa* types and multilocus sequence types belonging to the livestock-associated MRSA clones have been recently reported (*4*) and were not included in our study.

This cross-national prevalence study found livestock-associated MRSA ST398 in human samples in several European countries. However, the relatively low proportion of MRSA ST398 among MRSA isolates from humans in most countries suggests that MRSA ST398 contributes to only a small fraction of all MRSA in humans.

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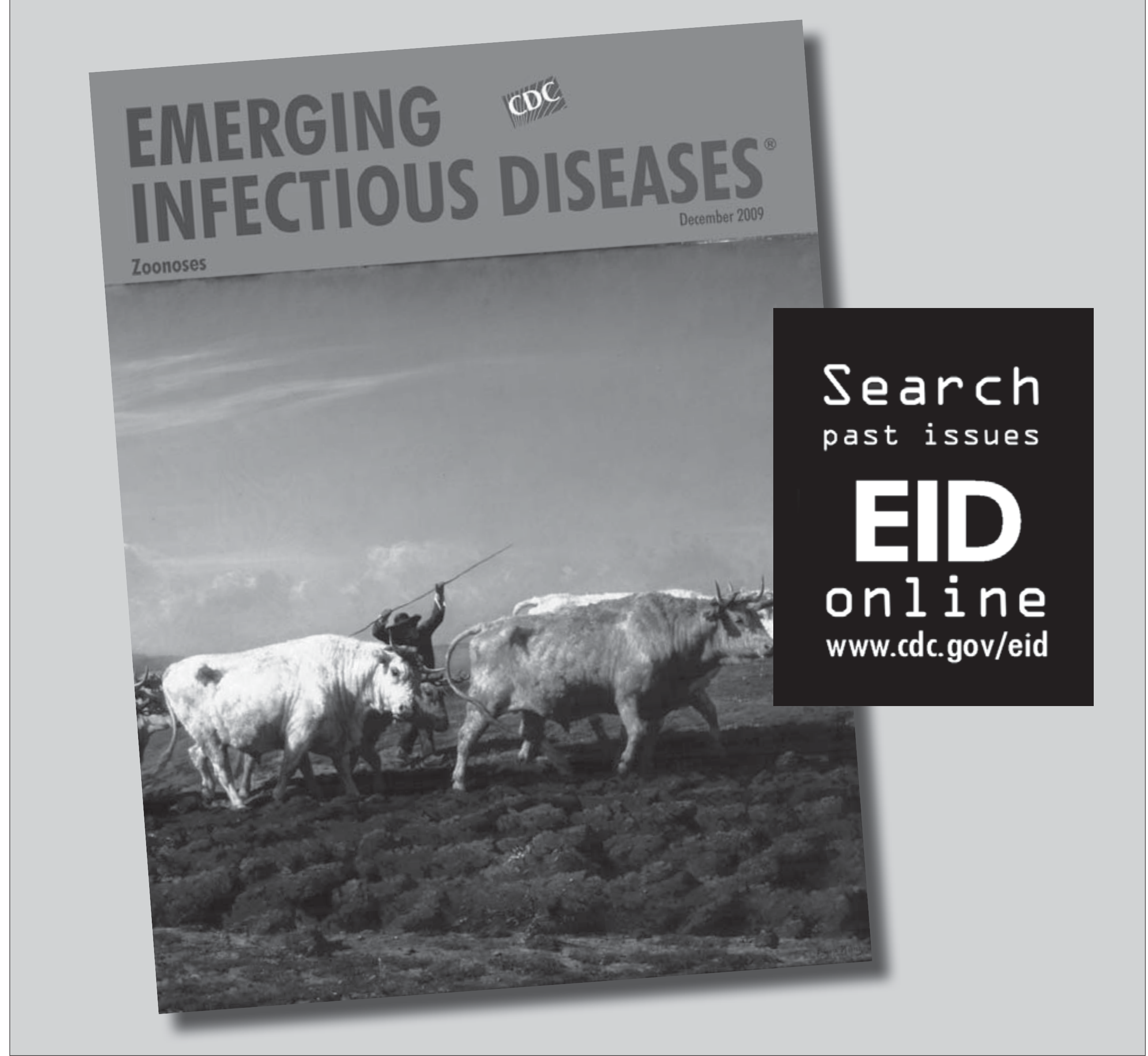

Distinguishing Tuberculosis from Nontuberculous Mycobacteria Lung Disease, Oregon, USA

Brian A. Kendall, Cara D. Varley, Dongseok Choi, P. Maureen Cassidy, Katrina Hedberg, Mary A. Ware, and Kevin L. Winthrop

To determine whether tuberculosis (TB) and nontuberculous mycobacteria (NTM) infection patients could be distinguished from one another with limited information, we compared pulmonary TB and NTM patients during 2005–2006. Our finding that age, birthplace, and presence of chronic obstructive pulmonary disease could differentiate TB and NTM disease could assist tuberculosis control efforts.

Patients seeking treatment who have respiratory specimens positive for acid-fast bacilli present a public health dilemma. Although *Mycobacterium tuberculosis* and nontuberculous mycobacteria (NTM) cause chronic lung infections, only tuberculosis (TB) spreads from person to person by inhalation of organisms expectorated into the air. NTM infections are acquired directly from the environment, where they are often present in soil and various water sources. The prevalence of NTM disease is reported to be increasing and is likely greater than that of TB in the United States (*1–3*). Because definitive identification of mycobacterial species can take several weeks, the ability to quickly distinguish NTM from TB on clinical grounds could help public health officials make decisions regarding contact investigations and isolation. To date, little population-based data exist that compare characteristics of pulmonary TB and NTM patients because previous studies have been limited to single institutions (*4–6*).

The Study

We identified patients reported to the Oregon Health Division with pulmonary TB during 2005–2006 who lived within the Portland metropolitan region (Clackamas, Multnomah, and Washington Counties). This region had a combined population of ≈1.55 million in 2005–2006 (*7*). In 2000, the predominant ethnicity in this region was white (75.8%), followed by Hispanic or Latino (11.4%), Asian (6.3%), and black (3.6%), and 11.9% of the population had been born outside the United States (*7*). From a statewide surveillance project, we identified all tri-county residents with NTM respiratory isolates obtained during the same period and then used pulmonary NTM disease criteria of the American Thoracic Society/Infectious Diseases Society of America to define cases of pulmonary NTM disease (*3*,*8*). For each pulmonary TB and NTM case-patient within the tri-county region, we collected demographic information. From physician records, we collected clinical data. We conducted this project under the authority of the Oregon Administrative Rules for special studies to control a public health problem.

We used SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) to compare categorical variables in univariate fashion by the χ^2 or Fisher exact tests. We calculated the relative proportion (RP) of TB patients with each risk factor compared to the proportion of NTM patients with the risk factor. We used the Student *t* test to evaluate continuous variables. We considered factors with a p value <0.2 for multivariate logistic regression and performed stepwise backward elimination of variables not reaching levels of statistical significance (p<0.05). Using significant variables from our multivariate model, we calculated the positive predictive value (PPV) and 95% exact binomial confidence intervals (CIs) of variables, alone and in combination, for distinguishing TB from NTM disease. Age was dichotomized (≤50 and >50 years) based on the age of NTM case-patients to simplify calculation of PPV (*9*).

Eighty-two pulmonary TB patients were reported; all but 2 had complete clinical records for review. We identified 407 patients with respiratory NTM isolates. Clinical records were present for 283 (69.5%) of these patients, of whom 127 (44.9%) met clinical criteria of the American Thoracic Society/Infectious Diseases Society of America for pulmonary NTM disease (*8*). Fifty-four patients lacked information on country of birth. In patients for whom smear data was available, no important difference was found in proportion of case-patients with smear-positive results (38/79 [46%] of TB case-patients vs. 28/47 [60%] of NTM case-patients). In comparison to NTM case-patients, TB patients were younger (median age 44 years, range 5–86 years vs. 67 years, range 12–92 years; p<0.01), more likely to be male (RP 1.6 , 95% CI 1.2–2.2, p<0.01), and more likely to have been born outside the United States (RP 4.0, 95% CI 2.5–6.3, p<0.01) (Table 1). *Mycobacterium avium-intracellulare* complex was the most common etiologic agent of NTM disease in our cohort (114 [90%]).

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Table 1. Demographic, clinical, and radiographic features of TB patients compared with NTM patients, Oregon, USA, 2005–2006*

Characteristic	No. (%) TB patients, n = 80†	No. (%) NTM patients, n = 127	Relative proportion (95% CI)	p value
Demographics				
Median age, y (range)	44 (5–86)	67 (12–92)	0.95 (0.93–0.96)	<0.01‡
Male	49 (61)	48 (38)	1.6 (1.2–2.2)	<0.01
Not US born§	65 (81)	15 (19)	4.0 (2.5–6.3)	<0.01‡
Clinical signs and symptoms				
Cough	58 (73)	98 (77)	0.9 (0.8–1.1)	0.45
Hemoptysis	12 (15)	28 (22)	0.7 (0.4–1.3)	0.21
Constitutional symptoms¶	56 (70)	61 (48)	1.5 (1.2–1.8)	0.03‡
Chest radiograph				
Bronchiectasis	2 (3)	6 (5)	0.5 (0.1–2.6)	0.71
Cavity	18 (23)	11 (9)	2.7 (1.3–5.3)	<0.01
Effusion	10 (13)	8 (6)	2.1 (0.9–5.0)	0.10
Infiltrate	68 (87)	69 (54)	1.6 (1.3–1.9)	<0.01‡
Lymphadenopathy	4 (5)	3 (2)	2.2 (0.5–9.4)	0.43
Concurrent conditions				
Immunosuppressive medications#	8 (10)	34 (27)	0.4 (0.2–0.8)	<0.01
COPD	2 (3)	29 (23)	0.1 (0.0–0.4)	0.19‡
Previous TB	3 (4)	13 (10)	0.4 (0.1–1.2)	0.11
Diabetes	10 (13)	8 (6)	2.0 (0.8–4.8)	0.12
Tobacco smoking (previous or current)	26 (33)	53 (42)	0.8 (0.5–1.1)	0.19
Lung cancer	4 (5)	8 (6)	0.8 (0.2–2.6)	0.77
HIV/AIDS	0	4 (3)		0.30

*TB, tuberculosis; NTM, nontuberculous mycobacteria; CI, confidence interval; COPD, chronic obstructive pulmonary disease.
†Two TB patients excluded because of missing clinical data, 4 from multivariate analysis (n = 78).
‡p value from multivariate analysis including COPD, age, not US born, constitutional symptoms, and infiltrate on radiograph.
§54 (26%) patients excluded because of missing country of origin.
¶Fever, night sweats, weight loss, or appetite loss.
#Systemic corticosteroids, inhaled corticosteroids, disease-modifying anti-rheumatic drugs, tissue necrosis factor-α inhibitors, cancer chemotherapy, and calcineurin inhibitors.

Clinically, TB patients were more likely to report constitutional symptoms (56 [70%] vs. 61 [48%], RP 1.5, 95% CI 1.2–1.8, p<0.01), less likely to have chronic obstructive pulmonary disease (COPD) (2 [3%] vs. 29 [23%], RP 0.1, 95% CI 0.0–0.4, p<0.01]), and less likely to be using immunosuppressive medications than NTM patients (8 [10%] vs. 34 [27%], RP 0.4, 95% CI 0.2–0.8, p<0.01) (Table 1). The most common immunosuppressive medications were systemic corticosteroids (30 patients [14%]). Patients with TB were more likely to have cavitation (18 [23%] vs. 11 [9%], RP 2.7, 95% CI 1.3–5.3, p<0.01) and infiltrate reported (68 [87%] vs. 69 [54%], RP 1.6, 95% CI 1.3–1.9, p<0.01) on chest radiograph (Table 1).

Birth outside the United States (odds ratio [OR] 26.3, 95% CI 9.9–69.6, p<0.01), constitutional symptoms (OR 3.0, 95% CI 1.1–8.0, p = 0.03), and infiltrate on chest radiograph (OR 7.8, 95% CI 2.6–23.9, p<0.01) were significantly associated with TB in multivariate analysis. Age was inversely related to the likelihood of having TB with an OR of 0.95 (95% CI 0.93–0.98, p<0.01) for each year increase in age. Because of its clinical significance, COPD (OR 0.3, 95% CI 0.1–1.7, p = 0.19) was maintained in the multivariate model. Four patients with missing covariate data were excluded (Table 1).

In our predictive model, age <50 years and birth outside the United States together were highly predictive for TB (PPV 0.98, 95% CI 0.88–1.0). COPD was poorly predictive of TB (PPV 0.06, 95% CI 0.01–0.21). Age >50, US-born status, and COPD together had a PPV for TB of 0.08, 95% CI 0.00–0.38 (Table 2; Figure).

Conclusions

In this population-based study comparing the demographic and clinical features of TB and NTM patients in a region of low TB incidence, we found that birthplace outside the United States, age, and the presence of COPD can accurately categorize 98% of patients in whom NTM disease is suspected. This information could be useful in making early isolation and treatment decisions in regions of low TB incidence.

According to recent surveillance data from the Centers for Disease Control and Prevention, 26 states had TB incidence similar to Oregon at ≤3 patients per 100,000 population; nationwide, 59% of patients were born outside the United States (*10*). With regard to the proportion of patients who were not born in the United States, and the proportion of the general population who were not born in the United States, Oregon is similar to many other states with a low-incidence of TB. Fourteen states with low TB

Table 2. PPVs of patient characteristics for tuberculosis in Oregon, USA, an area of low tuberculosis incidence, 2005–2006*

Variable	No. patients	No. TB cases	PPV for TB (95% CI)
Age <u><</u>50 y, not US born	44	43	0.98 (0.88–1.00)
Age >50 y, US born, COPD	12	1	0.08 (0.00–0.38)
COPD	31	2	0.06 (0.01–0.21)
Age <u><</u>50 y	70	49	0.70 (0.58–0.80)
Not US born	80	65	0.81 (0.71–0.89)
Infiltrate	137	68	0.50 (0.41–0.58)
Constitutional symptoms	117	56	0.48 (0.39–0.57)

*PPV using all patients (n = 207); tuberculosis cases = 80. PPV, positive predictive value; TB, tuberculosis; CI, confidence interval; COPD, chronic obstructive pulmonary disease.

incidence have >50% of TB cases occurring in non–US-born patients in a setting in which <12.3% of the total population is not born in the United States. Furthermore, 35 states had a similar racial composition to Oregon with a white, non-Hispanic population >72.6% (*7*). Oregon is therefore representative of many low-incidence TB areas within the United States.

Although the strength of this study is the population-based data, this circumstance also leads to limitations. The ratio of TB to NTM prevalence in a given geographic area likely varies, which affects the degree to which our results can be generalized. Unfortunately, NTM disease prevalence rates are largely unknown. Marras et al. reported a similar prevalence of NTM isolation in Ontario, Canada, but a higher incidence of TB (*11*). They also reported finding fewer *M. avium* complex and more *M. xenopi* and rapidly-growing mycobacteria (*11*). Regions less dominated by *M. avium* complex or with differing TB/NTM prevalence ratios might find different associations. Additionally, further analysis of patients with smear-positive results was precluded by inadequate sample size. A subgroup analysis of smear positive patients in a larger cohort would be useful.

In summary, we found that TB and NTM could be reliably differentiated by determining patient's birthplace, age, and presence of COPD. Until improved tools are developed for rapid mycobacterial diagnosis, these data might enable public health practitioners and clinicians in other regions with low TB incidence to plan more effective TB control efforts.

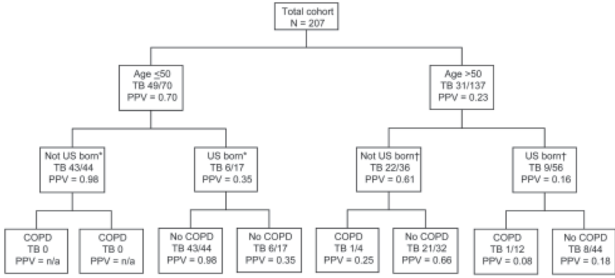

Figure. Positive predictive values (PPV) for tuberculosis of demographic and clinical factors in combination. TB, tuberculosis; COPD, chronic obstructive pulmonary disease; *9 patients missing birthplace; †45 patients missing birthplace.

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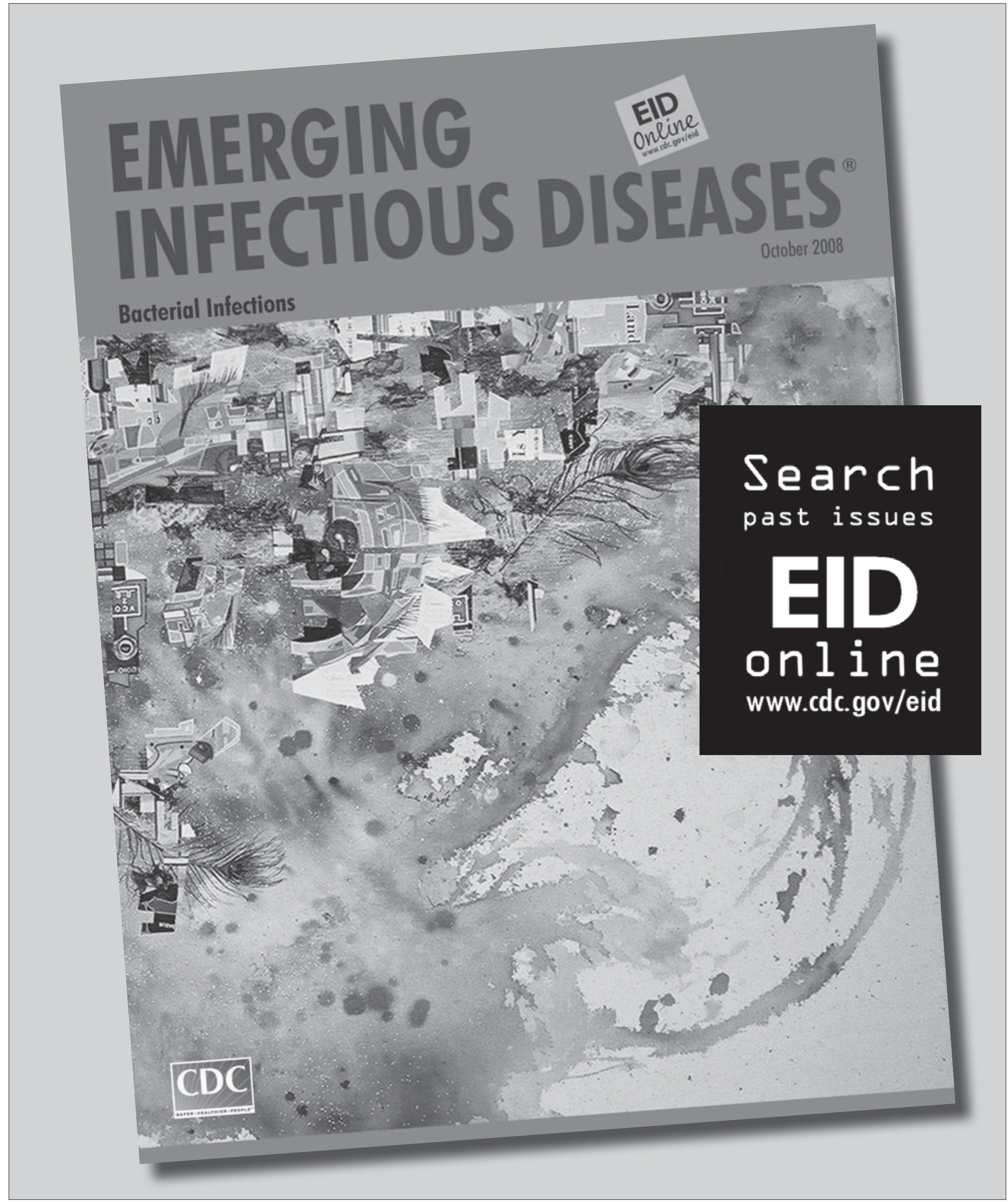

Increasing Drug Resistance in Extensively Drug-Resistant Tuberculosis, South Africa

N. Sarita Shah, Jessica Richardson, Prashini Moodley, Salona Moodley, Palav Babaria, Melissa Ramtahal, Scott K. Heysell, Xuan Li, Anthony P. Moll, Gerald Friedland, A. Willem Sturm, and Neel R. Gandhi

We expanded second-line tuberculosis (TB) drug susceptibility testing for extensively drug-resistant *Mycobacterium tuberculosis* isolates from South Africa. Of 19 patients with extensively drug-resistant TB identified during February 2008–April 2009, 13 (68%) had isolates resistant to all 8 drugs tested. This resistance leaves no effective treatment with available drugs in South Africa.

Extensively drug-resistant tuberculosis (XDR TB) was first reported in 2005 and has been identified worldwide (*1*,*2*). XDR TB is associated with poor treatment outcomes (*3*), especially among persons co-infected with HIV (*4*,*5*). XDR TB strains are created when multidrug-resistant TB (MDR TB) is inadequately treated, which enables amplification of second-line drug resistance (*6*,*7*). Inadequate treatment for XDR TB may result in additional resistance, severely limiting options for effective treatment.

Drug-susceptibility testing (DST) for first-line and second-line TB drugs is essential for developing effective MDR TB and XDR TB treatment regimens (*8*,*9*). However, DST requires laboratory facilities that are unavailable in most settings with high incidence of TB. In the last global report of drug resistance, only one third of countries conducted routine DST on suspected cases of TB; the remaining countries restricted testing to high-risk patients (*2*). Thus, most high-incidence settings use standardized MDR TB or XDR TB treatment regimens based on epidemiologic data from periodic drug-resistance surveys. Because the epidemiology of drug-resistant TB is changing rapidly (*10*), successful use of standardized regimens depends on accurate population-level drug-resistance data.

In 2005, a large HIV-associated XDR TB epidemic was detected in Tugela Ferry, South Africa (*5*). Continuous, routine drug resistance surveillance for all suspected TB cases has since been implemented in Tugela Ferry and >500 XDR TB cases have been diagnosed; >90% of patients are coinfected with HIV. Most early XDR TB isolates from Tugela Ferry were resistant to 4 or 5 drugs (isoniazid, rifampin, ofloxacin, and kanamycin, plus ethambutol or streptomycin) (*4*). XDR TB isolates have become resistant to an increasing number of drugs, such that by 2007, >90% were resistant to all 6 first-line and second-line drugs tested (Figure 1) (*4*).

DST for other second-line drugs (in addition to ofloxacin and kanamycin) is not routinely performed in South Africa. However, the standardized XDR TB treatment regimen includes second-line drugs for which drug resistance data are lacking. Thus, we sought to further characterize second-line TB drug resistance among XDR TB isolates in Tugela Ferry by expanding DST to include capreomycin and ethionamide.

The Study

This study was conducted in Tugela Ferry, South Africa, where TB incidence is ≈1,100 cases/100,000 population and >80% of TB case-patients are HIV infected.

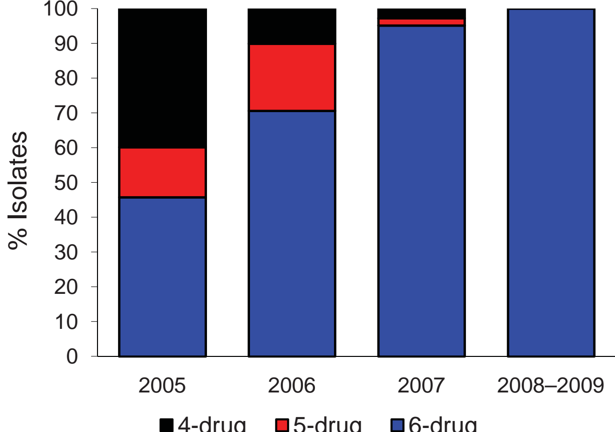

Figure 1. Drug resistance among extensively drug-resistant tuberculosis isolates from Tugela Ferry, South Africa, 2005–2009: 4-drug resistance = isoniazid (INH), rifampin (RIF), ofloxacin (OFL), and kanamycin (KM); 5-drug resistance = INH, RIF, OFL, KM, and ethambutol (EMB) or streptomycin (SM); 6-drug resistance = INH, RIF, OFL, KM, EMB, and SM. Column for 2008–2009 indicates study population.

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MDR TB and XDR TB incidence was 118 cases and 72 cases/100,000 population, respectively, in 2007 (*4*). Ethical approval for this study was obtained from Albert Einstein College of Medicine, Yale University, University of KwaZulu-Natal, and the KwaZulu-Natal Department of Health.

We performed a prospective cross-sectional study actively identifying patients with suspected TB in medical and TB wards, the HIV clinic, and the outpatient department at the Tugela Ferry district hospital during February 2008–April 2009. A person with suspected TB was defined as someone having a self-reported cough of any duration or ≥2 other signs or symptoms, including fever, night sweats, weight loss, or shortness of breath for any duration. Patients could be either newly manifesting TB symptoms or have been receiving TB treatment for ≥2 months but currently reporting active TB symptoms (i.e., treatment failures). One sputum specimen was obtained for culture and DST.

Sputum for this study was tested by microscopic analysis of auramine- and Ziehl-Nielsen–stained smears and Middlebrook 7H11 agar and Mycobacterial Growth Indicator Tube 960 broth culture. Identification of *Mycobacterium tuberculosis* was confirmed by using niacin and nitrate reductase tests. DST of positive cultures was performed by using the 1% proportional method on Middlebrook 7H11 agar for isoniazid (critical concentrations: isoniazid 0.2 µg/mL, rifampin 1.0 µg/mL, ethambutol 7.5 µg/mL, streptomycin 2.0 µg/mL, ofloxacin 2 µg/mL, kanamycin 5.0 µg/mL, capreomycin 10 µg/mL, and ethionamide 5.0 µg/mL). DST was repeated on all drug-resistant isolates to confirm the observed resistance pattern.

Medical records were reviewed for demographic and clinical data. The proportion of patients with XDR TB and drug-susceptibility patterns were described by using simple frequencies. XDR TB treatment outcomes were reported as of November 2009; standard international definitions were used (*11*).

Of 912 enrolled patients with suspected TB, 209 (23%) had culture-positive TB (Figure 2). Of these patients, 30 (14%) had MDR TB, of which 19 (63% of those with MDR TB; 9% with culture-positive results) had XDR TB.

Among XDR TB isolates, all 19 (100%) were resistant to all 6 drugs routinely tested in KwaZulu-Natal Province (isoniazid, rifampin, ethambutol, streptomycin, ofloxacin, and kanamycin), which extended the trend seen in previous years toward increasing drug resistance (Figure 1). Of these isolates, 4 (21%) were also resistant to capreomycin, and 13 (68%) were resistant to capreomycin and ethionamide (Table 1). Thus, an 8-drug resistance pattern was the predominant DST type among XDR TB patients in this cohort.

Of 13 patients with 8-drug resistance XDR TB, 5 (38%) were women (median age 33.5 years, range 24–51 years) (Table 2). Although 5 (38%) had previously received (or currently showed failure to) first-line TB treatment, none had ever received treatment with second-line drugs for MDR TB. Seven (54%) patients were identified from the ambulatory HIV clinic. Twelve (92%) patients were HIV infected (median CD4 cell count 183.5 cells/mm^3, range 22–670 cells/mm^3); only 2 (17%) were receiving antiretroviral therapy at the time of TB screening.

Among 13 XDR TB patients with 8-drug resistance, 7 (54%) died (median time to death 59 days, range 16–205 days). Two patients were lost to follow-up, and 4 (31%) are still living and receiving XDR TB treatment (range 190–502 days of follow-up). No trend in survival of patients with XDR TB was observed by drug-resistance pattern (6-drug vs 7-drug vs. 8-drug).

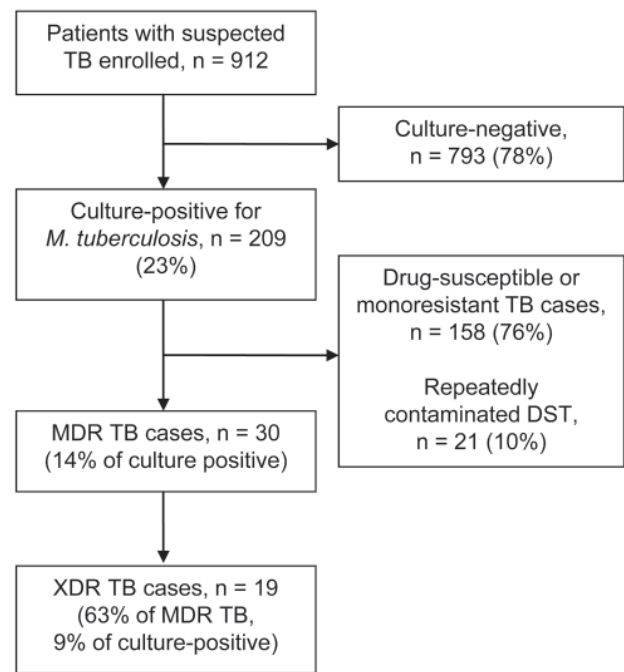

Figure 2. Determination of prevalence of tuberculosis (TB) and drug resistance among persons with suspected TB, Tugela Ferry, South Afica, 2008–2009. DST, drug susceptibility testing; MDR TB, multidrug-resistant TB; XDR TB, extensively drug-resistant TB.

Table 1. Drug susceptibility test results for 19 XDR TB patients, South Africa*

Drug resistance pattern (antibiogram)	No. (%) patients
INH, RIF, EMB, SM, OFL, KM	2 (11)
INH, RIF, EMB, SM, OFL, KM, CAP	4 (21)
INH, RIF, EMB, SM, OFL, KM, CAP, ETO	13 (68)
Total	19 (100)

*XDR TB, extensively drug-resistant tuberculosis; INH, isoniazid; RIF, rifampin; EMB, ethambutol; SM, streptomycin; OFL, ofloxacin; KM, kanamycin; CAP, capreomycin; ETO, ethionamide.

Table 2. Characteristics of 30 MDR TB and XDR TB patients, South Africa*

Characteristic	MDR TB	6- or 7-drug XDR TB	8-drug XDR TB
Total	11	6	13
Female sex	8 (73)	4 (67)	5 (38)
Age, y, median (range)	36 (26–52)	42.5 (36–64)	33.5 (24–51)
Prior TB treatment			
First-line drugs†	5 (45)	5 (83)	5 (38)
Second-line drugs‡	0	0	0
TB contact	2 (18)	2 (33)	1 (8)
Enrollment site at HIV clinic	8 (73)	2 (33)	7 (54)
HIV positive	10 (91)	5 (83)	12 (92)
CD4 cell count, cells/mm^3, median (range)	155 (25–708)	117.5 (18–426)	183.5 (22–670)
Receiving antiretroviral therapy (among HIV-positive patients)	7 (70)	2 (40)	2 (17)

*Values are no. (%) unless otherwise indicated. MDR TB, multidrug-resistant tuberculosis; XDR TB, extensively drug-resistant TB. 6-drug resistance, resistance to isoniazid, rifampin, ethambutol, ofloxacin, kanamycin, and streptomycin; 7-drug resistance, resistance to isoniazid, rifampin, ethambutol, ofloxacin, kanamycin, streptomycin, and capreomycin; 8-drug resistance, resistance to isoniazid, rifampin, ethambutol, ofloxacin, kanamycin, streptomycin, capreomycin, and ethionamide.
†First-line drugs used for treatment of persons with new TB cases or confirmed drug-susceptible TB include isoniazid, rifampin, ethambutol, and pyrazinamide.
‡Second-line drugs used for treatment of persons with confirmed MDR TB include ofloxacin, kanaymycin, ethionamide, *p*-aminosalicylic acid, and cycloserine or terizidone.

Conclusions

Routine drug-resistance surveillance to first- and second-line drugs is conducted in Tugela Ferry, which has a high incidence of TB and HIV co-infection. In this study, we expanded second-line testing for 2 additional bactericidal drugs (capreomycin and ethionamide) for treatment of patients with XDR TB. Resistance to 8 first-line and second-line drugs is the predominant pattern for XDR TB in Tugela Ferry, thereby severely limiting effective therapeutic options with available medications. According to the standard XDR TB regimen used in this province, patients were receiving ≤3 active drugs (pyrazinamide, *p*-aminosalicylic acid, and cycloserine), which increases the risk for treatment failure and further amplification of drug resistance. These findings underscore the need for routine surveillance for resistance to all first-line and second-line drugs used and for tailoring regimens accordingly to improve treatment success and reduce emergence of more drug-resistant XDR TB strains.

This study had 3 main limitations. First, the reliability of second-line DST is variable, and only recently have methods and critical concentrations been standardized (*12*). However, all drug-resistant isolates in this study had DST repeated to confirm observed results. Second, DST for other first-line drugs, such as pyrazinamide, and other second-line drugs was not conducted, although these drugs are often used for XDR TB treatment. Thus, the degree of drug resistance was likely to be only a minimum estimate. Third, although the proportion of XDR TB cases in this survey was high, the absolute number of XDR TB cases was low. This small sample size limits our ability to make conclusions about treatment outcomes for patients with increasing drug-resistant isolates. However, previous studies from our site have shown poorer survival rates with increasing drug resistance (*4*).

Expanded DST for second-line and third-line drugs is critical for XDR TB patient care. Given continued high and rapid number of deaths from XDR TB, better and more rapid methods for second-line DST are urgently needed to improve diagnosis and guide treatment. Although new drugs are being developed, efforts must target prevention of XDR TB and its transmission, earlier identification of cases, support of treatment completion for TB and MDR TB, and greater use of antiretroviral therapy for patients who are co-infected with HIV.

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Tuberculosis in Kindergarten and Primary School, Italy, 2008–2009

Antonietta Filia, Giuseppe Ciarrocchi, Rossana Belfiglio, Monaldo Caferri, Antonino Bella, Claudio Piersimoni, Daniela Cirillo, Gualtiero Grilli, Cristina Mancini, and Donato Greco

An outbreak of tuberculosis (TB) in Italy involved 19 schoolchildren with active TB and 43 with latent infection. The source of the outbreak was a school assistant born in Italy who had a family history of TB. This outbreak highlights the need for maintaining clinical and public health expertise in countries with low TB incidence.

The decrease in incidence of tuberculosis (TB) since the mid-1900s in Italy has resulted in loss of expertise in TB diagnosis, management, and control (*1*). However, TB is still present in Italy; each year >4,000 new cases are reported to the statutory reporting system (7.4 cases/100,000 population in 2008), and 46% of cases occur in foreign-born persons (*2*). As in other industrialized countries, cases in children are uncommon. Occurrence of TB in children indicates ongoing transmission of the disease and reflects effectiveness of a TB control program. Several TB outbreaks in schools have been reported in low-incidence countries in the past decade (*3–6*). We report an outbreak that occurred in a kindergarten/primary school in Italy.

The Study

A contact investigation was performed by local health authorities in central Italy after a report in November 2008 of culture-positive pulmonary TB in a 3-year-old girl. She attended a kindergarten in a building that housed 3 schools (kindergarten 1, kindergarten 2, and a primary school). Initially, children in the girl's class (kindergarten 1, class C), her family contacts, and some staff were tested. A high percentage (34.6%, 9/26) of classmates were positive for TB by Mantoux tuberculin skin test (TST), and health authorities decided to test all school children and staff. Contacts of persons with new cases identified were also tested. This contact investigation was part of a routine public health response to a school outbreak and did not require ethics committee approval.

Persons were screened by clinical evaluation, TST, and/or chest radiography. A positive TST result was defined as induration ≥5 mm (*7*). Persons with negative TST results were retested 10 weeks later. Children with positive TST results for whom a diagnosis of active TB had been ruled out were prescribed isoniazid for 6 months. Children <5 years of age were prescribed isoniazid regardless of TST results if active TB was ruled out. Those children with negative results at initial screening stopped treatment after 10 weeks if repeated testing showed negative results. Persons with chest radiograph abnormalities compatible with TB underwent further diagnostic evaluation.

A total of 851 persons, of whom 817 (96%) were born in Italy, were screened (Table 1). At initial testing, 53 (13.8%) of 383 children had positive TST results. Among 9 children with a positive result at follow-up, 1 had not undergone initial testing; 8 children showed skin test result conversion. Nineteen children had active TB (18 with pulmonary TB and 1 with extrapulmonary TB [Pott disease]). Forty-three children had latent TB infection (Table 2). No cases of active TB were identified among school staff and contacts of children with positive TST results.

In January 2009, a case of smear-negative, culture-positive pulmonary TB with cavitary involvement of both upper lung lobes was reported to the same local health authorities by a hospital in southern Italy. The case-patient was a 42-year-old woman born in Italy who worked as an assistant in kindergarten 1 and was considered the presumed source of the outbreak. She reported a family history of TB (son and father treated for pulmonary TB in 1996 and 1999, respectively) and positive TST results, but did not recall completing a chemoprophylaxis regimen with isoniazid. She also reported persistent cough for 1 year, which was treated with mucolytic and antimicrobial drugs by her family physician. During this time, she had frequent contact with many children, especially in kindergarten 1, and occasionally worked as an assistant in kindergarten 2. She had a negative TST result during the school contact investigation; no chest radiograph was prescribed. She subsequently traveled to southern Italy, where she consulted a respiratory physician who requested immediate hospitalization.

Gastric aspirate specimens were obtained from 18 children, and a vertebral abscess pus specimen was obtained from 1 child. Sputum specimens were obtained from the source case-patient. Ziehl-Neelsen microscopy, direct amplification tests, cultures, and drug-susceptibility

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Table 1. Results of initial and follow-up screening for tuberculosis, Italy, 2008–2009*

Persons screened by TST or chest radiograph	No. tested	Median age, y (range)	TST induration ≥5 mm, no. positive/no. tested (%)		No. positive/no. with negative TST result at initial screening (%)
			Initial screening	Follow-up testing	
Schoolchildren	388	6 (2–11)	53/383 (13.8)	9/320 (2.8)	320/330 (97.0)
School staff	77	45 (24–60)	15/71 (21.1)	7/53 (13.2)	53/56 (94.6)
Family contacts†	207	37 (1–89)	35/203 (17.2)	10/90 (11.1)	90/168 (53.6)
Casual contacts	179	29 (2–66)	18/173 (10.4)	4/106 (3.8)	106/155 (68.4)
Total	851	8 (1–89)	121/830 (14.6)	30/569 (5.3)	569/709 (80.2)

*All categories are mutually exclusive. TST, tuberculin skin test.
†Household and immediate family members of children with positive skin test results. Does not include family contacts of the source case-patient.

testing were performed according to standard procedures. Specimens from 3 children were positive for acid-fast bacilli, and specimens from 9 children were culture positive for *Mycobacterium tuberculosis*. All isolates were susceptible to first-line drugs against TB except 1 that was moderately resistant to isoniazid. DNA fingerprinting showed that the strain isolated from the source case-patient belonged to the S family lineage and was identical to all strains isolated from children in this study.

Median age of children with active TB was 5 years (9 were girls and 15 had signs or symptoms). Attack rates were significantly higher for children in kindergarten 1 than for those in kindergarten 2 and the primary school ($p<0.001$) (Table 2). Differences in attack rates in 4 classes of kindergarten 1 were not significant. In the primary school, high attack rates were found among children in grade 1; no active TB cases and few children with positive TSTs results were found in higher grades ($p<0.001$). Most children in grade 1, including the 3 with active TB, were in kindergarten 1 the preceding school year. All children except 1 (lost to follow-up) have completed treatment and are healthy.

Conclusions

This outbreak indicates TB transmission in a low-incidence country. Genotyping results confirmed that all cases were linked to the school assistant, and clinical and epidemiologic data suggest that she was the only source of infection. Infected children in grade 1 were most likely infected the preceding school year. Young children with TB are not usually infectious. During this outbreak, it is unlikely that they transmitted TB to others. The major factor that contributed to the outbreak was the delay in diagnosis of the source case, as in other reported outbreaks (*3*–*6*). This finding indicates the need for increasing awareness of TB among primary care physicians and for improving their ability to recognize risk factors for infection and progression to active disease. Also, if the school assistant had received appropriate treatment and follow-up at the times her son and father received diagnoses of TB, active disease might never have developed. This finding highlights the importance of appropriate follow-up of contacts in response to a case of TB.

Our study has several limitations. Urgency was required because of the school setting, pressure by parents, and media coverage. As in other school-contact investigations, this urgency may have led to excessive screening of low-risk persons and difficulties in categorizing contacts by close and casual status (*8*). Also, information regarding risk factors for TB and previous TST results was not consistently recorded. Nevertheless, the investigation had a high rate of test completion among children and staff.

Table 2. Attack rates for children with latent and active tuberculosis in 2 kindergartens and a primary school, Italy, 2008–2009*

School class and grade	No. students	Median age, y (range)	No. latent TB/no. tested (%)	No. active TB/no. tested (%)	No. infected/no. tested (%)
Kindergarten 1	107	4 (3–6)	16/103 (15.5)	13/103 (12.6)	29/103 (28.2)†
Class A	23	4 (4–6)	2/22 (9.1)	4/22 (18.2)	6/22 (27.3)
Class B	28	5 (4–6)	7/27 (25.9)	4/27 (14.8)	11/27 (40.7)
Class C	28	4 (3–4)	5/26 (19.2)	4/26 (15.4)	9/26 (34.6)
Class D	28	3 (3–3)	2/28 (7.1)	1/28 (3.6)	3/28 (10.7)
Kindergarten 2	82	4 (2–6)	3/81 (3.7)	3/76 (3.9)	6/81 (7.4)
Primary school	199	8 (5–11)	24/199 (12.1)	3/192 (1.6)	27/199 (13.6)
Grade 1	45	6 (5–8)	16/45 (35.6)	3/45 (6.7)	19/45 (42.2)†
Grade 2	29	7 (6–7)	3/29 (10.3)	0/26 (0.0)	3/29 (10.3)
Grade 3	40	8 (7–9)	1/40 (2.5)	0/38 (0.0)	1/40 (2.5)
Grade 4	47	9 (9–11)	2/47 (4.3)	0/47 (0.0)	2/47 (4.3)
Grade 5	38	10 (9–10)	2/38 (5.3)	0/36 (0.0)	2/38 (5.3)
Total	388	6 (2–11)	43/383 (11.2)	19/371 (5.1)	62/383 (16.2)

*TB, tuberculosis.
†$p<0.001$ by χ^2 test or, when appropriate, Fisher exact test or χ^2 test for trend.

Although TB among foreign-born persons is of increasing importance in many industrialized countries because of immigration from high-incidence areas, this outbreak highlights that TB is of concern not only for those of foreign origin (*2*). TB was once a common disease in Italy, and a pool of improperly treated persons may be at risk for reactivation of TB. A high proportion of school staff had positive TST results, probably because many were born at a time when the risk for TB was high. In Italy, there are no specific requirements regarding TB screening of school employees. When screening is initiated in a low-prevalence population, it should focus on high-risk persons, and priority should be given to quality surveillance, follow-up, and containment activities. However, consideration should be given to administering a health questionnaire to school staff (foreign and native born) who have sustained contact with small children to identify high-risk persons, as is being conducted elsewhere in Europe (*3,9*).

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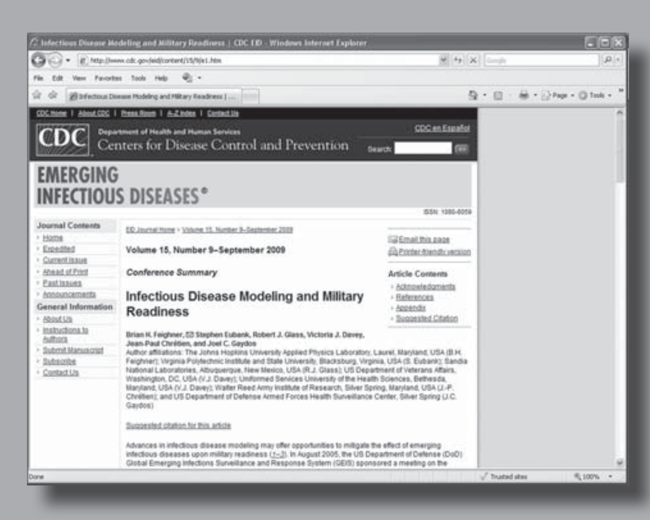

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Tuberculosis among Foreign-born Persons, Singapore, 2000–2009

Khin Mar Kyi Win, Cynthia B.E. Chee, Liang Shen, Yee T. Wang, and Jeffery Cutter

We determined the proportion of foreign-born persons with tuberculosis (TB) in Singapore. This proportion increased from 25.5% in 2004 to 37.6% in 2009. Unskilled workers from countries with high incidences of TB accounted for the highest number of and greatest increase in foreign-born TB case-patients.

Singapore, an island city-state (area 710 km^2) in Southeast Asia, liberalized its immigration policy and underwent rapid economic growth during 2005–2010. This policy resulted in a marked increase in its population from 4.17 million in 2004 to 5.08 million in 2010, which was largely caused by an increase in foreign-born persons comprising long-term pass holders (LTPHs) (permission to stay in Singapore >6 months), permanent residents (PRs), and naturalized citizens (*1*). In recent decades, mass immigration and influx of nonimmigrants from countries with high incidences of tuberculosis (TB) to industrialized countries have contributed to the epidemiology and incidence of TB in host countries (*2*–*9*). We report the epidemiology of TB in foreign-born case-patients in Singapore during 2000–2009.

The Study

Notification of TB cases to the Singapore TB Elimination Programme (STEP) Registry is mandated by law. Since 1998, the notification report has included disease characteristics, sociodemographic information, country of origin, immigration status, and year of arrival in Singapore of TB patients (*10*). All notifications during 2000–2009 were obtained from the STEP Registry database. When data on country of birth were not available for persons not born in Singapore (0.5% of case-patients), the nationality of these persons was assumed to represent their country of birth. Missing information about country of birth for persons with a Singaporean nationality (17%) and arrival date (6.5%) were matched against a database provided by the Ministry of Home Affairs.

Persons not born in Singapore were considered foreign born. Foreign-born case-patients comprised naturalized citizens, PRs, and LTPHs. Singapore issues long-term passes to skilled and unskilled workers and others (students and foreign-born family members of citizens or PRs) to enable a stay >6 months in Singapore. Persons applying for permanent residency or long-term stay and renewal of long-term passes are given medical examinations that include a general physical examination, chest radiograph, and testing for HIV (*11*). The Department of Singapore Statistics provided 2000–2010 population data. We estimated the number of persons born in Singapore during 2000–2010 by using linear interpolation based on 2000 and 2010 population census exercises. Consistent with the country classification used in the Singapore 2000 population census, People's Republic of China, Hong Kong, and Taiwan were classified as 1 group of country of birth.

There were 23,164 cases of TB (new and re-treated) reported to the registry during 2000–2009. After we excluded 4,033 short-term pass holders, persons whose TB diagnosis had changed (n = 97), and persons with missing information about country of origin (n = 34), a total of 19,000 persons were eligible for the study. Of these persons, 13,048 (68.7%) were born in Singapore and 5,952 (31.3%) were foreign born. The number and proportion of foreign-born cases decreased in the first half of the study period from 675 (33.6%) in 2000 to 444 (25.5%) in 2004. This trend reversed during 2005, and the number and proportion of foreign-born persons with TB increased to 788 (37.6%) in 2009 (Figure 1). Of foreign-born persons with TB, 3,386 (56.9%) were LTPHs, 1,820 (30.6%) were PRs, and 746 (12.5%) were naturalized citizens.

LTPHs with TB comprised 2,562 (75.7%) unskilled workers, 371 (10.9%) professional and skilled workers, and 162 (4.8%) students. The remaining 291(8.6%) persons were foreign-born family members of Singapore citizens or PRs who were granted long-term stay in Singapore. TB among LTPHs increased from 220 (49.5%) cases in 2004 to 532 (67.5%) in 2009 and that among PRs increased from 45 (6.7%) in 2000 to 124 (15.7%); cases among foreign-born citizens decreased from 342 (50.7%) in 2000 to 132 (16.8%) in 2009 (Figure 2, panel A).

The number of persons with TB from Indonesia, the Philippines, and Myanmar increased from 2000–2004 to 2005–2009 (Figure 2, panel B). Among native-born persons, TB occurred predominantly in men >40 years of age (Table). TB in foreign-born persons was distributed equally in both sexes and occurred mostly in persons 20–39 years of age (Table). Drug resistance and extrapulmonary involvement were higher among foreign-born persons than among native-born persons.

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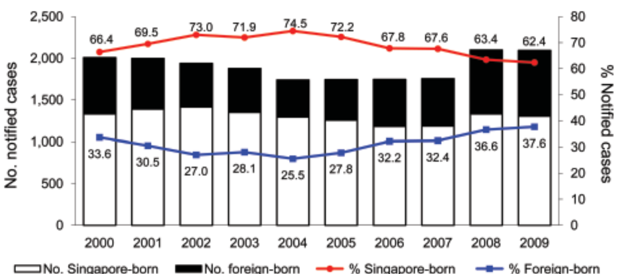

Figure 1. Tuberculosis (TB) cases and proportion of native-born versus foreign-born persons, Singapore, 2000–2009. Numbers along data lines indicate percentage of native-born persons with TB versus foreign-born persons with TB.

Conclusions

The decreasing trend of foreign-born persons with TB in the first half of the study period could be attributed to fewer transient migrant workers entering Singapore during the economic crisis in Asia and the severe acute respiratory syndrome outbreak in 2003. After economic recovery and liberalization of the immigration policy in Singapore in 2005, there was an influx of migrant workers and immigrants from countries with high incidences of TB and a corresponding increase in TB notifications among this population. Of these persons with TB, >75% came from 5 of the 7 countries (India, China, Indonesia, Bangladesh, and the Philippines) with highest incidences of TB (*12,13*).

The large increase in the number of TB case-patients from Indonesia, the Philippines, and Myanmar reflects the influx of migrant workers from these countries. This finding is consistent with the predominance of cases reported in foreign-born persons 20–39 years of age. The lower incidence of diabetes among these younger persons than in native-born persons is not surprising. The preponderance of lymph node involvement among the foreign-born persons merits further study.

The estimated TB rate in persons born in Singapore decreased from 49.7 cases/100,000 population to 41.8/100,000 during 2000–2007 but increased to 46.5/100,000 in 2008 and 45.2/100,000 in 2009. This increase followed the increase in cases among foreign-born persons in 2005. Transmission from foreign-born to native-born persons may have occurred, especially in increasingly crowded urban settings of Singapore. However, this proposal cannot be verified without DNA fingerprinting studies.

Currently, persons with untreated inactive lesions or scarring on their screening chest radiographs are given permission for a long-term stay in Singapore without further examination or follow-up. To improve screening, mandating sputum TB cultures may be prudent for these persons. If active disease is excluded, these persons can then receive prophylactic treatment.

We do not favor a policy of screening for latent TB infection because of the high probability of false-positive tuberculin skin test results in persons vaccinated with *Mycobacterium bovis* BCG and the unfavorable risk-benefit ratio of preventive therapy for persons from countries with high TB incidences, who may be more likely to have acquired latent TB in the past than recently (*14*). Although interferon-γ release assays will overcome the problem of false-positive tuberculin skin test results in persons vaccinated with *M. bovis* BCG, the cost of these assays is prohibitively high, and their positive predictive value for progression to active TB remains to be determined (*15*).

A strength of this study was its nationally representative data of all TB cases reported to the STEP Registry. Electronic linkage of the Registry to the 2 mycobacterial culture laboratories in Singapore also enabled inclusion of all bacteriologically positive cases. A limitation of this study was the unavailability of data for native-born and foreign-born persons by sex and age to compare age- and sex-specific TB case rates between these 2 groups. In addition, because of the lack of population data for country of birth, we were unable to determine the TB rate by country of origin. Also, information bias is inherent in retrospective studies, and most country of birth data were based on information submitted by the notifying physician. We were also unable to analyze the association of HIV and TB because access to HIV status in our study population

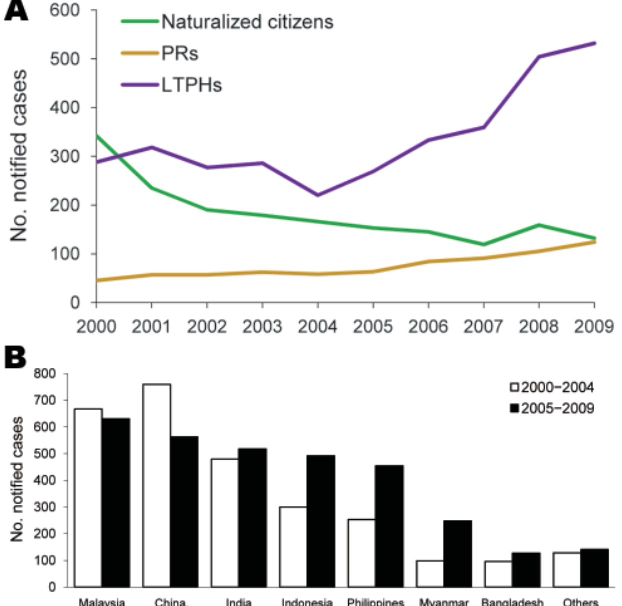

Figure 2. Tuberculosis (TB) cases, Singapore, 2000–2009. A) No. notified cases among foreign-born subgroups, by year of notification. Citizens, naturalized citizens; PRs, permanent residents; LTPHs, long-term pass holders. B) No. notified cases by country of origin.

Table. Characteristics of native-born and foreign-born persons with TB, Singapore, 2000–2009*

Characteristic	No. (%) native-born, n = 13,048	No. (%) foreign-born, n = 5,952
Sex		
F	3,546 (27.2)	2,731 (45.9)
M	9,502 (72.8)	3,221 (54.1)
Age, y		
Median	53.2	33.8
0–19	516 (4.0)	149 (2.5)
20–39	2,592 (19.9)	3,503 (58.9)
40–59	5,137 (39.4)	812 (13.6)
≥60	4,803 (36.8)	1,488 (25.0)
Diabetes		
No	10,282 (78.8)	5,409 (90.9)
Yes	2,766 (21.2)	543 (9.1)
New case	11,550 (88.5)	5,588 (93.9)
Re-treated case	1,498 (11.5)	364 (6.1)
Site of disease		
Pulmonary†	11,447 (87.7)	4,832 (81.2)
Extrapulmonary	1,601 (12.3)	1,120 (18.8)
Site of extrapulmonary TB‡		
Lymph node	571 (35.7)	582 (52.0)
Pleura	415 (25.9)	258 (23.0)
Other	698 (43.6)	328 (29.3)
Sputum smear for AFB		
Negative	5,744 (44.0)	2,790 (46.9)
Positive	4,784 (36.7)	1,532 (25.7)
Not determined	2,520 (19.3)	1,630 (27.4)
Sputum culture for MTC		
Negative	3,075 (23.6)	1,819 (30.6)
Positive	8,316 (63.7)	2,855 (48.0)
Not determined	1,657 (12.7)	1,278 (21.5)
Drug resistance		
MDR§	28 (0.3)	39 (1.4)
Isoniazid¶	256 (3.1)	226 (7.9)

*TB, tuberculosis; AFB, acid-fast bacilli; MTC, *Mycobacterium tuberculosis* complex; MDR, multidrug resistant.
†Includes pulmonary TB cases with extrapulmonary involvement.
‡Pure extrapulmonary and not mutually exclusive.
§Resistant to at least isoniazid and rifampin.
¶Includes MDR; resistant to isoniazid alone or isoniazid in combination with any other first-line drugs.

was limited. Our study highlights the need for measures to address TB among foreign-born persons in Singapore.

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Phylogeny of European Bat Lyssavirus 1 in *Eptesicus isabellinus* Bats, Spain

Sonia Vázquez-Morón, Javier Juste, Carlos Ibáñez, José M. Berciano, and Juan E. Echevarría

To better understand the epidemiology of European bat lyssavirus 1 (EBLV-1) in Europe, we phylogenetically characterized *Lyssavirus* from *Eptesicus isabellinus* bats in Spain. An independent cluster of EBLV-1 possibly resulted from geographic isolation and association with a different reservoir from other European strains. EBLV-1 phylogeny is complex and probably associated with host evolutionary history.

The genus *Lyssavirus* comprises 3 species that can infect bats in Europe: *European bat lyssavirus 1* (EBLV-1), *European bat lyssavirus 2*, and *West-Caucasian bat virus* (*1,2*). Most lyssavirus-infected bats have been found in north-central Europe (Germany, the Netherlands, Denmark, Poland, and France); of these, >95% were serotine bats (*Eptesicus serotinus)* infected by EBLV-1 (*3–5*). EBLV-1 in other bat species has rarely been described (*3,6*). EBLV-1–infected bats become increasingly scarce from north to south in Europe, and no cases in northern Spain or Italy have been reported. The same trend has been consistently found within Germany (*3*) except for an artifact that arose from varied surveillance intensity among different countries. However, several infected serotine bats in southern Spain have been reported (*7*). These bats have been assigned to the species *E. isabellinus*, which has closely related populations on the African side of the Gibraltar Strait (*8*). This species is strongly divergent from *E. serotinus* bats (>16% of cytochrome b gene) in the northern Iberian Peninsula (*9*). In Spain, the distribution of EBLV-1 cases in bats apparently coincides with the distribution of *E. isabellinus* bats; 10 cases of human exposure after contact with infected bats have been reported; each was associated with *E. isabellinus* bats.

Two subtypes have been proposed for EBLV-1: EBLV-1a, which extends from the Netherlands to Russia in a west–east axis, and EBLV-1b, which includes strains that extend south through France and the Netherlands and the only 2 published strains from Iberia (*1*). We phylogenetically characterized EBLV-1 strains associated with *E. isabellinus* bats, a reservoir in the Iberian Peninsula that differs from *E. serotinus* bats.

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The Study

We sequenced 12 bat brains positive for *Lyssavirus* antigen detected by immunofluorescence and reverse transcription–PCR (RT-PCR) as described (*10*). All viruses were identified as EBLV-1. For phylogenetic analyses, the 400-bp 5′ variable extreme of the nucleoprotein gene of these EBLV-1 strains was amplified by specific EBLV-1 nested RT-PCR and sequenced by using the following primers: SEQVAR1F 5′-$_{1}$ACGCTTAACAACCAGATCAAAG$_{22}$-3′, SEQVAR2F 5′-$_{51}$AAAAATGTAACACYYCTACA$_{70}$-3′, EBLVSEQVAR1R 5′-$_{596}$CAGTCTCAAAGATCTGTTCCAT$_{575}$-3′, and EBLVSEQVAR2R 5′-$_{552}$TAGTTCCCAGTATTCTGTCC$_{533}$-3′.

All rabies-positive serotine bats came from southern Spain (Huelva, Seville, Murcia, and Badajoz) and were molecularly identified as *E. isabellinus* (*8*). An alignment was performed by using ClustalX (www.clustal.org) to combine the obtained sequences and other available EBLV-1 sequences from GenBank, including a Duvenhage virus used as the outgroup (online Appendix Table, www.cdc.gov/EID/content/17/3/520-appT.htm). Before conducting further analyses, we used jModel Test (http://darwin.uvigo.es/software/jmodeltest.html) to select the best fitting substitution model for our sequences according to the corrected Akaike information criterion. Maximum-likelihood phylogenies were reconstructed by using PHYML (http://atgc.lirmm.fr/phyml) software and by using a generalized time-reversible model and the γ parameter estimated in the analyses. Maximum-parsimony analyses were conducted by using PAUP* 4.0b10 (http://paup.csit.fsu.edu/) weighting transversions 15× according to the transitions/transversion ratio estimated in the jModelTest analyses. Confidence in the topologies for the maximum-likelihood and the maximum-parsimony analyses was established with 1,000 bootstrap replicates. A Bayesian phylogenetic inference was obtained by using MrBayes version 3.1 (http://mrbayes.csit/fsu.edu/) with random starting trees without constraints. Two simultaneous runs of 10^7 generations were conducted, each with 4 Markov chains, and the trees were sampled

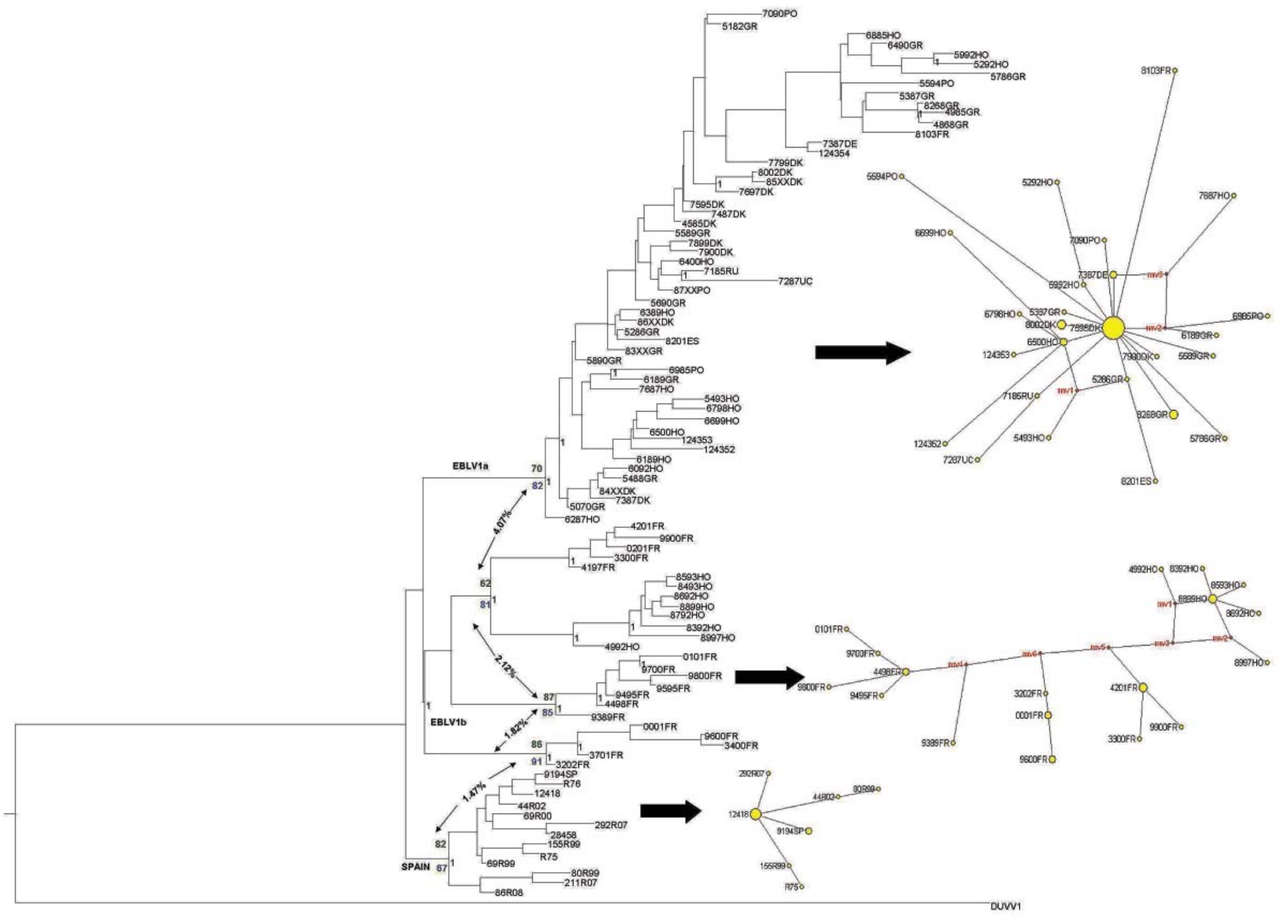

Figure 1. European bat lyssavirus 1 (EBLV-1) phylogenetic reconstruction based on the first 400 bp of the nucleoprotein gene. The tree was obtained by Bayesian inference run for 10^7 generations; trees were sampled every 100 generations. The first 25% of trees were excluded from the analysis as burn-in. Black numbers indicate posterior probabilities. Bootstrap supports after 1,000 replicates for each node are also shown for maximum-parsimony (green numbers) and maximum-likelihood (blue numbers) analyses. Net *p*-distance values (as percentages) between groups are indicated by arrows. A parsimony-based network is presented for each major lineage; sizes of yellow circles are proportional to the number of individuals sharing a given haplotype, and reconstructed haplotypes (median vectors) are shown in red. DUVV, Duvenhage virus.

every 100 generations. Net p-distances between groups were calculated by using MEGA4 (www.megasoftware.net/) (Figure 1).

The genetic structure and relationships between haplotypes were examined within the main lineages through a parsimony-based network built with a median-joining algorithm implemented in the Network 4.5.1 program (*11*). To evaluate and compare genetic variability and polymorphism among lineages, we estimated the number of haplotypes, mutations, and segregating sites as well as haplotype diversity and nucleotide diversity by using DNAsp version 4.5 (*12*) for the major clades (Table). Finally, to investigate population dynamics across lineages, the Fu F*s* and Tajima D statistics were calculated (Table).

Table. Genetic diversity statistics for EBLV-1*

Population	n	S	Eta	Hap	Hd	VarHd	Pi	ThetaNuc	k	Tajima D	Fu F*s*
EBLV-1a	52	45	48	26	0.836	0.00267	0.00664	0.02656	2.6546	–2.5693 (0.00000)	–21.676 (0.00000)
EBLV-1b	25	35	35	18	0.970	0.00038	0.02202	0-02317	8.8067	–0.1885 (0.48000)	–4.555 (0.05100)
EBLV-1Spain	13	9	9	7	0.795	0.01191	0.00538	0.00725	2.1538	–1.0138 (0.18100)	–2.067 (0.06143)

*EBLV, European bat lyssavirus; n, no. sequences; S, no. segregating sites; Eta, no. mutations; Hap, no. haplotypes; Hd, haplotype diversity; VarHd, haplotype variance; Pi, nucleotide diversity; ThetaNuc, estimated population mutation rate per site; k, average no. nucleotide differences; and neutrality tests (Tajima D and Fu F*s*).

Figure 2. Geographic distribution of *Eptesicus serotinus* bats (red), *E. isabellinus* bats (blue), and cases of rabies in bats (dots), Europe, 1990–2009. Obtained from Rabies Bulletin Europe (www.who-rabies-bulletin.org/).

These 2 statistics are considered to be the most powerful tests for detecting expansion events (*13*).

Conclusions

All phylogenetic analyses, regardless of the reconstruction criterion used, formed a monophyletic cluster of the EBLV-1 strains from Spain (only the Bayesian inference reconstruction is shown). The Bayesian inference, maximum-likelihood, and maximum-parsimony analyses identified the cluster from Spain and EBLV-1a and EBLV-1b as being monophyletic (Figure 1), although only maximum-likelihood and maximum-parsimony analyses suggested a closer relationship between EBLV-1b and the cluster from Spain. The genetic differentiation of the EBLV-1 strains from the Iberian Peninsula matches their association with another bat species (Figure 2), which suggests that the host bat's evolutionary history plays a major role in EBLV-1 molecular epidemiology, as has been proposed for rabies virus in bats in North America (*14*).

The low genetic diversity and the Fu F*s* and Tajima D statistics (Table) all suggest rapid population expansion of EBLV-1a, which is consistent with the star-like structure of the network for this lineage (Figure 1). Conversely, haplotype and nucleotide diversity descriptors (Table) have the highest values for EBLV-1b and a complex network structure with differentiated subnetworks. All these elements indicate that this lineage has a complex evolutionary history. The lineage from Spain also has low diversity and a star-shaped network, but neutral evolution cannot be rejected on the basis of the F*s* and D statistics. Net distances are similar within and between lineages, except for EBLV-1a, which is slightly more differentiated (Figure 1). Consequently, the suggested EBLV-1 expansion from Spain into Europe (*15*) is not supported by our results, which record the highest variability and most complex phylogenetic structure for France and the Netherlands (Figure 1). This complex structure suggests either a longer evolutionary history in these areas or a recent contact of distinct bat lineages in this zone.

The results of this study show that the strains from Spain do not belong to subtype 1b because of their association with a different reservoir (*E. isabellinus* bats). Moreover, what is currently considered to be EBLV-1b seems to include at least 4 lineages that are more genetically diverse and have a complex history. EBLV-1a, however, has low genetic diversity despite its extensive geographic distribution, suggesting a relatively recent and successful expansion of this lineage. These results call into question the current classification of EBLV-1 into 2 single subtypes. To provide a better understanding of EBLV-1 molecular epidemiology in Europe, additional studies that consider different genes should be conducted and the current classification should be revised accordingly.

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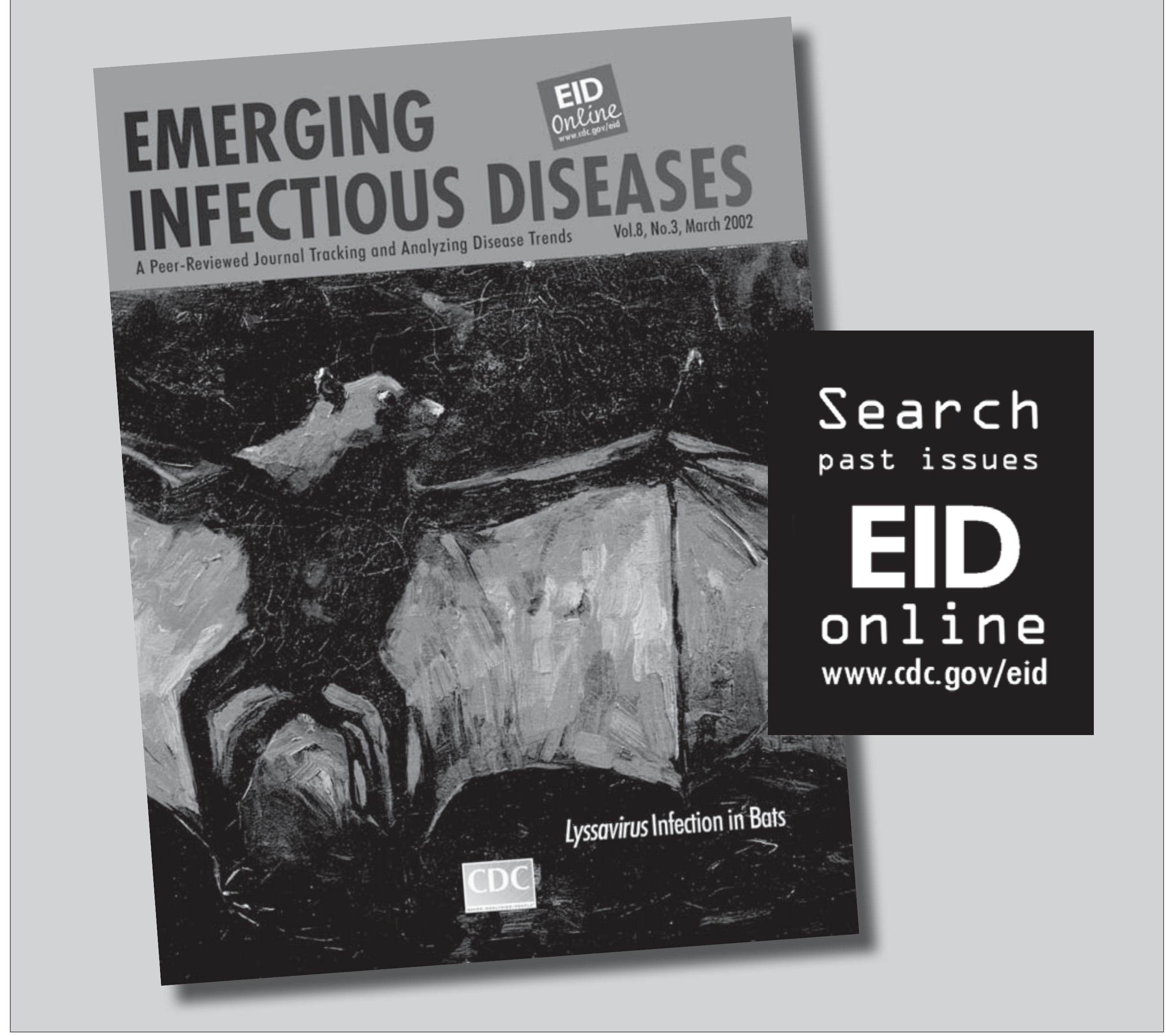

Escherichia coli O157 Infection and Secondary Spread, Scotland, 1999–2008

Mary E. Locking, Kevin G.J. Pollock, Lesley J. Allison, Linda Rae, Mary F. Hanson, and John M. Cowden

To determine the proportion of *Escherichia coli* O157 cases in Scotland attributable to secondary spread, we analyzed data obtained through entire-population enhanced surveillance. We identified 11% of cases as secondary. Secondary cases in single households were younger than secondary cases in outbreaks affecting >1 household and had similar risk for hemolytic uremic syndrome.

Escherichia coli O157 remains a substantial public health challenge worldwide, particularly because of its association with hemolytic uremic syndrome (HUS) (*1*,*2*). The low infectious dose (*3*) exacerbates its potential to cause secondary spread and large outbreaks (*4–10*). Most published information about secondary spread derives from outbreaks (*7*,*9–12*) or from subgroups or settings not necessarily generalizable to whole populations (*4*,*11*). Rates of secondary cases range from 4% to 16% (*4*,*9*).

Associations with increased transmission include presence of siblings, young age of persons with primary or potential secondary cases (*4*,*10*,*11*), and waterborne compared with foodborne transmission in outbreaks (*12*). Scotland consistently reports higher rates of *E. coli* O157 infection than many other countries (Figure 1); *E. coli* O157 was identified in 81% of HUS cases (*13*). Although large outbreaks have occurred (*7*,*8*), most infections in Scotland are apparently sporadic (*14*) (Figure 2).

The Study

In 1999, Health Protection Scotland (HPS), in close collaboration with the Scottish *E. coli* O157/VTEC Reference Laboratory (Edinburgh, Scotland), established enhanced surveillance of *E. coli* O157 covering the entire population. HPS defines a case as a single-person infection episode with laboratory confirmation of infection as either culture positive or serum positive for *E. coli* O157. The term case refers to symptomatic and asymptomatic persons, i.e., patients and nonpatients.

HPS compiles standardized datasets for all cases, integrating microbiologic, epidemiologic, demographic, and exposure data; datasets include direct clinical reports of HUS under enhanced surveillance of thrombotic microangiopathies since 2003 (*13*) and detailed symptom descriptions since 2004. Secondary cases are defined as those symptomatic cases from whose onset date and an assumed incubation period (≤14 days) we can infer that contact with a confirmed case was more likely than any other exposure to be the source of infection, and whose onset was >2 days after onset in the contact case. Cases having onset within 2 days after onset in a contact case are defined as co-primaries.

HPS surveillance systems collect information about general outbreaks, i.e., those affecting members of >1 household or residents of an institution. Other cases are therefore either sporadic or occur among members of a single household. For ease of comprehension, we refer to cases in general outbreaks as outbreak cases and cases or clusters restricted to a single household as sporadic cases, irrespective of whether they are secondary or primary cases. We analyzed data for all cases reported to HPS during 1999–2008 using χ^2 and Mann-Whitney tests and considered $p<0.05$ as significant.

From January 1, 1999, through December 31, 2008, a total of 2,228 *E. coli* O157 cases were reported to HPS (mean 223 annually); the mean annual incidence rate was 4.4 cases per 100,000 population (Table 1; Figure 1). Ages of the 2,228 cases ranged from 4 months to 97 years (median 21 years). A minority of all cases (202/2,228 [9%]) were asymptomatic, in similar proportions annually ($p = 0.44$) (Table 1). All 1,118 cases reported 2004–2008

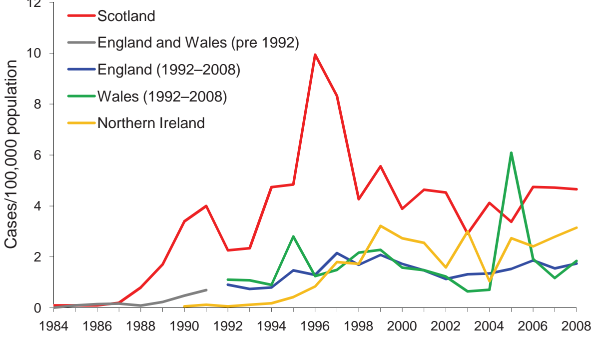

Figure 1. Rates per 100,000 population of laboratory-confirmed culture-positive *Escherichia coli* O157 cases, by country, United Kingdom, 1984–2008. Data outside Scotland courtesy of Health Protection Agency London, and Public Health Agency Belfast; figures for England, Wales, and Northern Ireland are verotoxin-positive cases only. Data for 2008 outside Scotland are provisional.

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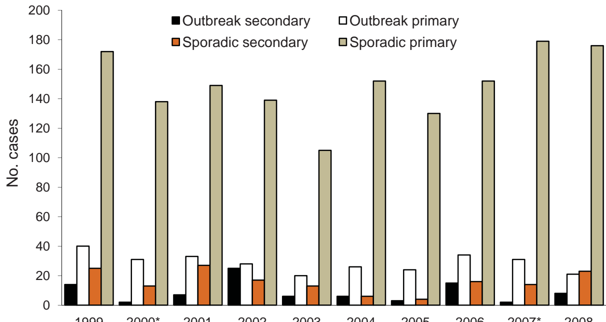

Figure 2. Secondary symptomatic or primary symptomatic laboratory-confirmed *Escherichia coli* O157 cases, by outbreak or sporadic occurence, Scotland, 1984–2008. *Only 2 cases were identified in this year.

provided symptom details; 660 (59%) had bloody diarrhea. Varying proportions of cases each year were hospitalized (mean 41%; p<0.005) or had illness progressing to HUS (196/2,228; mean 9%; p = 0.03) (Table 1). HUS occurred more often in cases reporting bloody diarrhea than nonbloody diarrhea (14% vs. 3%, p<0.0005), suggesting that bloody diarrhea may be a better predictor of progression to HUS than was previously apparent in Scotland.

Secondary cases constituted 246/2,026 (12%) of the symptomatic cases (11% of all cases), with proportions varying annually (range 4%–20%; p<0.0005) (Table 1; Figure 2), apparently independent of incidence rates. Secondary cases were younger than primary cases (median 13 years vs. 20 years; p<0.0005). Fewer secondary than primary cases had bloody diarrhea (54% vs. 66%; p = 0.02) (Table 2), but secondary cases with bloody diarrhea were younger than primary cases with bloody diarrhea (median 13 years vs. 26 years; p<0.03), perhaps reflecting lower thresholds for screening younger contacts. Secondary cases accounted for 12% of all HUS cases, and their likelihood of having HUS was similar to that of primary cases (p = 0.95) (Table 2). Mean time between onset in primary and secondary cases was 8 days (range 3–24 days); the longer times occurred when primary cases were symptomatic for >14 days. Child-to-child transmission accounted for 72% of secondary cases, child-to-adult for 19%, and adult-to-adult for 9%.

Most cases (1,787/2,228 [80%]) were sporadic (Table 1; Figure 2). Similar proportions of sporadic and outbreak cases (p = 0.89) had illness progressing to HUS. Of the 1,650 sporadic cases who were symptomatic, 158 (10%) were secondary cases (Table 2). Sporadic and outbreak secondary cases had the same risk for HUS (p = 0.97), but sporadic secondary cases were younger (median 9 years vs. 26 years; p<0.04), highlighting the need to prevent transmission within single households.

Outbreak cases constituted 441/2,228 (20%) cases, but proportions varied annually (range 13%–27%; p<0.0005) (Table 1; Figure 2). Of the 88 secondary cases in outbreaks, 57 (65%) lived in the same household as the associated primary case; the remainder were contacts either in institutions or in linked second households. The 441 outbreak cases comprised 104 separate outbreaks. Secondary cases were identified in 40 (42%) of the 95 outbreaks that occurred in Scotland, with an average ratio of secondary to primary cases of 1.3:1.

Conclusions

The reasons for high incidence rates of *E. coli* O157 in Scotland are undoubtedly complex and multifactorial.

Table 1. Selected characteristics and health outcomes of laboratory-confirmed *Escherichia coli* O157 cases, Scotland, 1999–2008*

Characteristic	No. (%) cases, n = 2,228†	Range per year, % (p value)
Average reports per year, all cases	223	153–282
Average annual incidence per 100,000 population	4.4	3.0–5.6
Symptomatic cases	2,026 (91)	89–94 (0.44)
Asymptomatic cases	202 (9)	6–11 (0.44)
Secondary case‡	246 (12)‡	4–20 (<0.0005)
Primary case‡	1,780 (888)‡	80–96 (<0.0005)
Bloody diarrhea§	660 (59)§	56–62 (0.80)
Hospitalized case-patients	902 (41)	29–48 (<0.0005)
Hemolytic uremic syndrome	196 (9)	6–14 (0.03)
Outbreak case	441 (20)	13–27 (<0.0005)
Sporadic case	1,787 (80)	73–87 (<0.005)
Sporadic and symptomatic¶	1,650 (92) ¶	90–95 (0.84)
Sporadic, symptomatic, and secondary#	158 (10)#	3–15 (<0.005)
Sporadic, symptomatic, and primary#	1,492 (90)#	85–97 (<0.005)

*Cases include symptomatic and asymptomatic persons.
†Unless otherwise indicated.
‡Denominator = 2,026 symptomatic cases 1999–2008.
§Denominator = 1,118 cases reported 2004–2008, all with symptom details available (1,022 symptomatic and 96 asymptomatic cases).
¶Denominator = 1,787 sporadic cases.
#Denominator = 1,650 symptomatic sporadic cases.

Table 2. Selected characteristics and health outcomes of secondary or primary symptomatic laboratory-confirmed *Escherichia coli* O157 cases, Scotland, 1999–2008

Characteristic	No. (%) cases, n = 2,026		p value
	Secondary cases, n = 246*	Primary cases, n = 1,780*	
Case age <10 y	116 (47)	623 (35)	<0.0005
Female sex	147 (60)	958 (54)	0.07
Bloody diarrhea†	52 (54)†	608 (66)†	0.02
Hospitalized	82 (33)	816 (46)	<0.0005
Illness progressed to hemolytic uremic syndrome	24 (10)	172 (10)	0.95
Sporadic case	158 (64)	1492 (84)	<0.0005
Outbreak case	88 (36)	288 (16)	<0.0005
Outbreak case, with bloody diarrhea‡	25 (28)‡	81 (28)‡	0.93
Outbreak case, hospitalized‡	32 (36)‡	128 (44)‡	0.22

*Unless otherwise indicated.
†Denominator = 1,022 symptomatic cases reported 2004–2008, all with symptom details available (97 secondary and 925 primary cases).
‡Denominator = 376 outbreak symptomatic cases (88 secondary and 288 primary cases).

Influences affecting real incidence may include the relative population densities of livestock and humans and reliance on private water supplies (*8*).

Ascertainment of secondary cases in Scotland, which appeared to have a greater role in our study than may have been commonly assumed previously, may however be particularly affected by artifactual influences, such as more assiduous contact tracing resulting from heightened awareness, perhaps triggered by a combination of large outbreaks, a national task force, and enhanced surveillance (*7*,*8*). This possibility necessitates caution in extrapolating our findings on secondary case incidence to other countries. Such tracing and confirmation of infection is valuable in controlling household transmission as well as outbreaks, and for clinical management (*10,11*). Alternatively, some secondary cases will undoubtedly be missed, or misclassified as primary cases.

Because most secondary cases in Scotland are apparently sporadic, our findings also reinforce the need for low thresholds for suspecting infectious etiology in acute diarrhea (particularly if bloody), irrespective of (and without waiting to discover) whether cases are part of outbreaks (*2*,*11*). Patients need immediate advice about infection control in the home, accompanied by immediate stool sampling and monitoring (e.g., blood parameters), not just for primary or index cases but also for their contacts (*9–11*). We must continually raise professional and public awareness of secondary spread and measures needed to reduce it, and to ameliorate health outcomes (*2*,*9*,*10*). We should also maintain preventive strategies targeting the livestock-related risks strongly associated with sporadic infection (*8*,*14,15*).

We believe enhanced surveillance in Scotland provides uniquely valuable information, particularly about secondary transmission, because data derive from the entire population and standardized and long term. They remove reliance on extrapolating from studies of outbreaks, subgroups, or other countries, which may use substantially different methods and settings. Our data also permit robust long-term analysis, which is central to identifying whether differences in incidence or epidemiology are real or artifactual, and whether those differences are meaningful for public health.

We strongly recommend increased efforts to prevent secondary transmission within individual households. This would reduce not only the overall health and social costs of *E. coli* O157 infection but also the number of, and distress to, HUS cases attributable to secondary spread.

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Ms Locking is an epidemiologist at Health Protection Scotland, where she established the national VTEC Enhanced Surveillance System. Her work includes investigating verotoxin-producing *E. coli* outbreaks and using surveillance data on the etiology and outcomes of infection to identify and support health protection initiatives, particularly in rural settings.

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Molecular Epidemiology of *Mycobacterium tuberculosis*, Buenos Aires, Argentina

Ximena Gonzalo, Marta Ambroggi, Ezequiel Cordova, Tim Brown, Susana Poggi, and Francis Drobniewski

To analyze the molecular epidemiology of *Mycobacterium tuberculosis* strains at a hospital in Buenos Aires, Argentina, and mutations related to multidrug-resistant and extensively drug-resistant tuberculosis, we conducted a prospective case–control study. Our findings reinforce the value of incorporating already standardized molecular methods for rapidly detecting resistance.

During the 1990s, an outbreak of multidrug-resistant (MDR) tuberculosis (TB) in HIV-positive patients occurred at the Muñiz Hospital in Buenos Aires, Argentina (*1*). Molecular analysis showed that a member of Haarlem2 family of *Mycobacterium tuberculosis* was responsible (*1*).

We conducted a prospective case-control study during June 1, 2006–April 30, 2007, at this 300-bed public hospital, which reports ≈40% of new TB cases in the city (*2*). Our primary aims were to analyze the molecular epidemiology of *M. tuberculosis* strains circulating at the hospital and the mutations related to MDR TB and extensively drug-resistant TB.

The Study

The strains were isolated and tested for antimicrobial drug susceptibility according to the proportion method (*3*) at the Muñiz Hospital Mycobacteria Laboratory. A proportion were tested for reserve drugs at the Health Protection Agency National Mycobacteria Reference Unit Laboratory, London, UK. DNA was extracted from cultures at this UK laboratory; spoligotyping was performed according to the manufacturer's instructions (Isogen Life Science, IJsselstein, the Netherlands); and data were analyzed with SPOTCLUST (http://cgi2.cs.rpi.edu/~bennek/SPOTCLUST.html).

We performed 15-locus variable number tandem repeat (VNTR) using a CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc., Fullerton, CA, USA) (*4*). Cluster analysis was performed by using Bionumerics software (Applied Maths, St-Martens-Latem, Belgium). Strains lacking a unique pattern were subjected to further analysis with an expanded set of VNTR loci (*5*).

In-house macroarrays were performed on MDR TB strains (*6*) to identify mutations in the *katG* and the *inhA* genes. Two regions of the *rpoB* gene of MDR TB strains were sequenced with the CEQ 8000 Genetic Analysis System. Statistical analyses were conducted by using χ^2 and Fisher exact tests.

After we excluded duplicates, treatment follow-ups, and strains with susceptibility patterns other than MDR TB or susceptibility to all drugs tested, 881 strains were susceptible to all drugs tested. Patients with a minimum dataset (name, sex, date of birth or age, TB presentation [i.e., pulmonary or nonpulmonary], and at least 1 sign or symptom describing TB illness and treatment received) were enrolled: 57 of 62 hospitalized patients with MDR TB cultures (Table) and 100 fully susceptible unmatched inpatient controls, for a total of 157 patients. This convenience sample (Figure 1) included only admitted patients because of the difficulty of obtaining clinical information about outpatients.

The most common spoligofamilies were T1 (18%), Haarlem 1 (10%), 2 (16%), and 3 (11%), LAM 3 (10%), and LAM 9 (13%). Initial 15-loci VNTR analysis showed 73 strains with unique patterns. Further analysis using 7-loci VNTR was performed on those that did not have a unique pattern. Twenty-six isolates remained clustered (Figure 2).

Of the 57 MDR TB strains, 43 had a mutation in the *katG315* locus, and 6 had a mutation in the *inhA* region. No strain had mutations in both genes. In 8 strains, resistance to isoniazid was not mediated by mutations in any of them.

Mutations in the *rpoB* region were detected by sequencing. The most frequent mutation was the S531L. Mutations at ≥1 site were rare. In 3 cases, we found 2 point mutations in the same codon. Only 1 MDR TB strain had no mutation in the *rpo*B segment sequenced; it also had a *kat*G and *inh*A wild type. Complete susceptibility profile for all 57 MDR TB strains is available in the online Appendix Table (www.cdc.gov/EID/content/17/3/528-appT.htm).

In addition, we detected 5 extensively drug-resistant TB strains. None were clustered by 22-loci VNTR typing.

Conclusions

Spoligotyping identified predominance of the Haarlem family among the MDR TB cases (family responsible for

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the 1990s [*1*] outbreak) as well as the LAM and T families. A similar strain family distribution was reported for the French Departments of the Americas (*7*) and Turkey (*8*). The Beijing family was seldom encountered in these areas, which is in line with recent observations in 7 countries in South America, including Argentina (*9*).

The MDR TB Haarlem2 strain appears to be more successful than other circulating MDR TB strains and than its susceptible counterpart (of 25 Haarlem2 strains, 20 were MDR TB). This phenomenon could be associated with a bias in the sample resulting from the specialized nature of the hospital or it could be that the MDR TB version has become predominant in the population because of the low fitness cost of its 2 mutations, *kat*G315 and S531L (*10,11*). In addition, the presence of clusters suggests that even though new technologies have reduced the time taken to diagnose drug resistance, more rapid initial diagnosis of MDR TB to reduce transmission still is needed (*12*).

All except 1 of the *rpo*B mutations in the MDR TB strains were at nt positions 1303–1375. This finding reinforces the value of incorporating already standardized molecular methods for rapidly detecting resistance. Cost

Table. Demographic information for 157 patients in a study of the molecular epidemiology of TB, Buenos Aires, Argentina, June 1, 2006–April 30, 2007*

Demographic characteristic	Patients with MDR TB, n = 57, no. (%)†	Patients with non–MDR TB, n = 100, no. (%)‡	p value, OR (95% CI)§
Sex			
M	35 (61)	70 (70)	0.271, 1.4667 (0.7404–2.9055)
F	22 (39)	30 (30)	
Location			
Buenos Aires area	46 (81)	95 (95)	0.004, 4.5435 (1.491–13.845)
Other	11 (19)	5 (5)	
Country of birth			
Argentina	43 (75)	66 (66)	0.2176, 0.632 (0.3041–1.3133)
Bolivia	6 (11)	20 (20)	
Peru	7 (12)	8 (8)	
Paraguay	0	3 (3)	
Uruguay	1 (2)	1 (1)	
Chile	0	1 (1)	
Missing data	0	1 (1)	
Education			
Illiterate or some primary	16 (28)	32 (32)	0.2059, 0.5185 (0.1860–1.4456)
Some secondary or tertiary	7 (12)	27 (27)	
Missing data	34 (59)	41 (41)	
Occupation			
Unemployed	7 (12)		
Construction and manual worker	20 (35)		
Factory worker	4 (7)	14 (14)	
Health care worker	1 (2)	1 (1)	
Education, i.e., student and teacher	2 (4)	4 (4)	
Housewife	6 (11)	5 (5)	
Missing data	17 (30)	23 (23)	
HIV infection			
Positive	25 (44)	27 (27)	0.04, 0.4737 (0.2308–0.9722)
Negative	25 (44)	57 (57)	
Missing data	7 (12)	16 (16)	
Nature of TB contact			
Close (i.e., household, family, co-worker)	10 (18)	32 (32)	
Institution (i.e., hospital, prison)	2 (4)	3 (3)	
Casual (e.g., acquaintance)	5 (9)	3 (3)	
Missing data	40 (70)	62 (62)	
TB presentation			
Pulmonary	36 (63)	61 (61)	0.7184, 1.1553 (0.5323–2.5073)
Nonpulmonary	15 (26)	22 (22)	
Missing data	6 (11)	17 (17)	

*TB, tuberculosis; MDR, multidrug resistant; OR, odds ratio; CI, confidence interval; IQR, interquartile range.
†Median age, y (IQR) for patients with MDR TB: 34 (27–40).
‡Median age, y (IQR) for patients with non-MDR TB: 28.5 (23.0–37.0).
§χ^2 test.

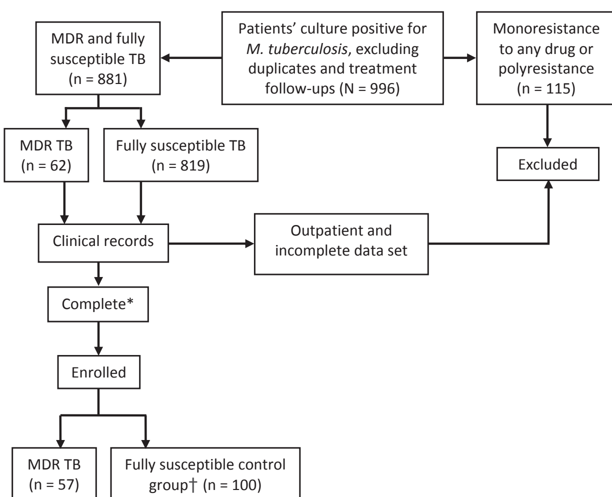

Figure 1. Multistage cluster sampling method for a study of the molecular epidemiology of *Mycobacterium tuberculosis*, Buenos Aires, Argentina, June 1, 2006–April 30, 2007. *Complete, having all but one of the following data: name, gender, date of birth or age, tuberculosis (TB) presentation, ≥1 sign or symptom describing the presentation and treatment received. †Unmatched controls. MDR, multidrug-resistant.

is the main reason they are currently not available, but the macroarrays used in this project are inexpensive and have the additional advantage of analyzing *kat*G and *inh*A mutations independently.

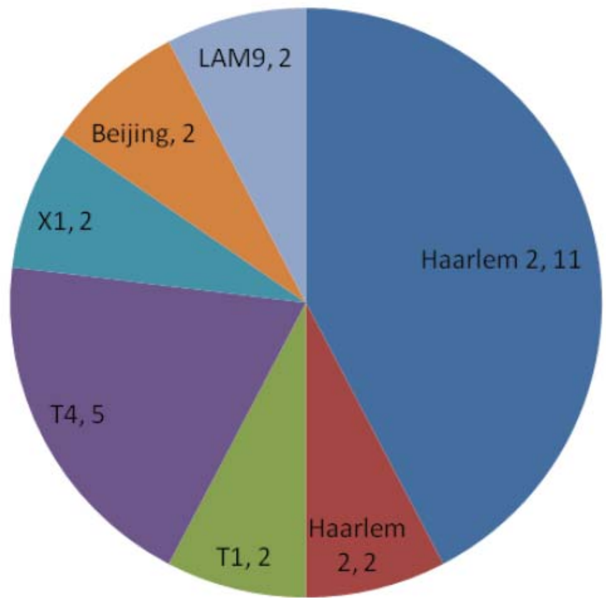

Figure 2. Final cluster results in a study of the molecular epidemiology of *Mycobacterium tuberculosis*, by spoligofamily, Buenos Aires, Argentina, June 1, 2006–April 30, 2007.

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Dr Gonzalo is a clinician and microbiologist working in the private and public health sector in Argentina on HIV, tuberculosis, and clinical microbiology. Her research interests include tuberculosis, mycobacteria, molecular epidemiology, and HIV.

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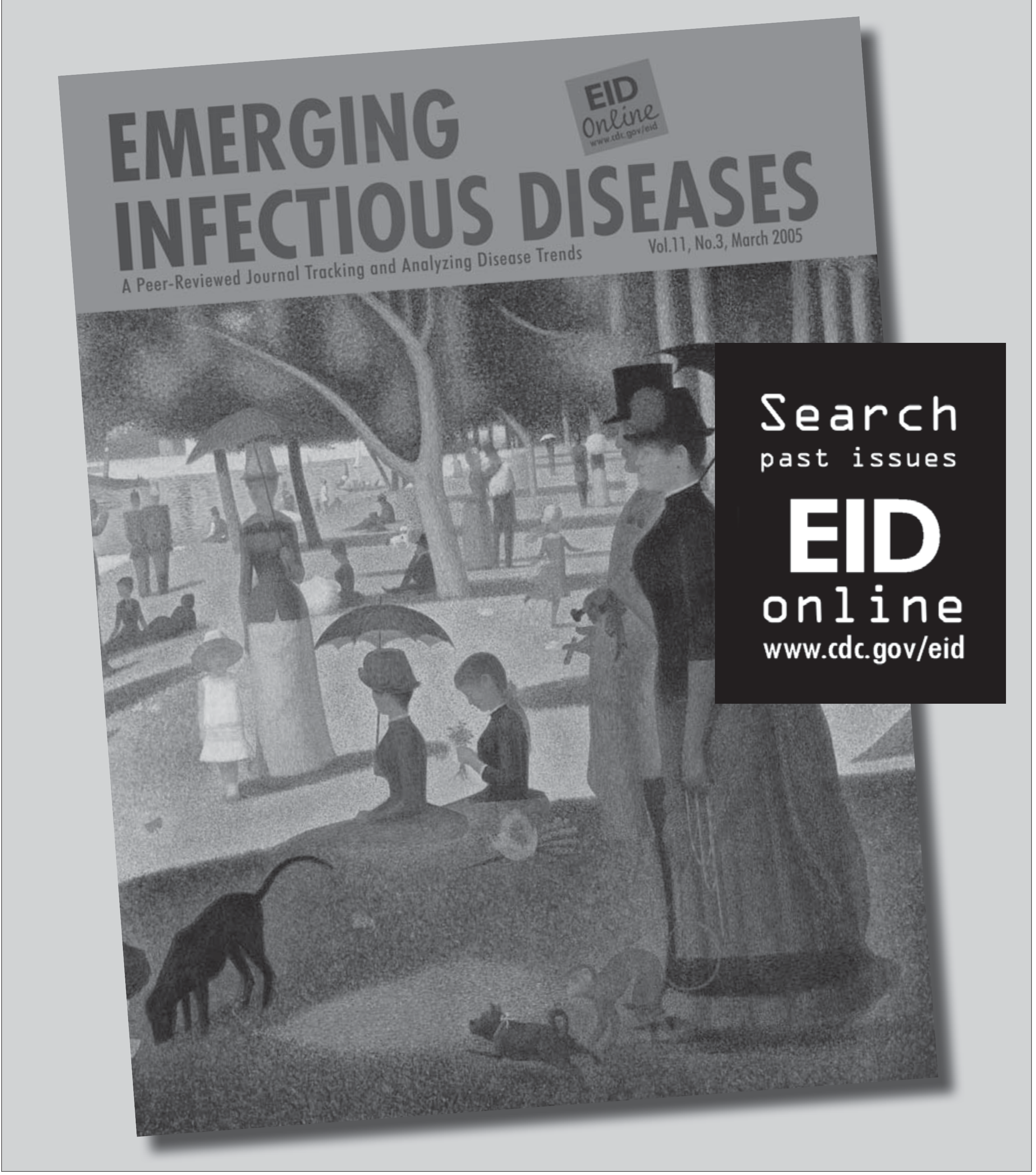

Mycobacterium caprae Infection in Livestock and Wildlife, Spain

Sabrina Rodríguez, Javier Bezos, Beatriz Romero, Lucía de Juan, Julio Álvarez, Elena Castellanos, Nuria Moya, Francisco Lozano, M. Tariq Javed, José L. Sáez-Llorente, Ernesto Liébana, Ana Mateos, Lucas Domínguez, Alicia Aranaz, and The Spanish Network on Surveillance and Monitoring of Animal Tuberculosis[1]

Mycobacterium caprae is a pathogen that can infect animals and humans. To better understand the epidemiology of *M. caprae*, we spoligotyped 791 animal isolates. Results suggest infection is widespread in Spain, affecting 6 domestic and wild animal species. The epidemiology is driven by infections in caprids, although the organism has emerged in cattle.

Mycobacterium caprae is a cluster within the *M. tuberculosis* complex (online Technical Appendix, www.cdc.gov/EID/content/17/3/532-Techapp.pdf). This pathogen has been recognized mainly in central Europe, where it has been occasionally isolated from tuberculous lesions from cattle (*1*–*5*), pigs (*4*), red deer (*Cervus elaphus*) (*4*,*5*), and wild boars (*Sus scrofa*) (*3*). Its isolation from humans has also been described (*3*,*6*); often, a contact with livestock has been suggested as a likely means of transmission (*5*). To our knowledge, this pathogen has never been isolated outside continental Europe, except from a European patient in Australia (*7*) and a cow in Algeria (*8*).

The combination of disease tracing and molecular typing is needed to understand the epidemiology of tuberculosis. This report describes the molecular epidemiology of *M. caprae* infection in Spain compared with other countries. We characterized *M. caprae* isolates from goats and other domestic and wild animals by spoligotyping (*9*). The relative contribution of each animal and its role in animal tuberculosis are discussed.

The Study

This study included 791 *M. caprae* isolates from domestic goats (*Capra aegagrus hircus*, n = 542), sheep (*Ovis aries*, n = 2), cattle (*Bos taurus*, n = 229), domestic pigs (*S. scrofa domestica*, n = 2), wild boars (*S. scrofa*, n = 14), red deer (*Cervus elaphus*, n = 1), and a fox (*Vulpes vulpes*, n = 1). The samples originated from skin test–positive animals identified within the national or regional eradication programs, from abattoir surveillance, and from postmortem inspections of wildlife, and were collected from 1992 through June 2009 in different geographic areas in Spain (Figure 1). Spoligotyping was performed as described (*9*), and authoritative names for spoligotype

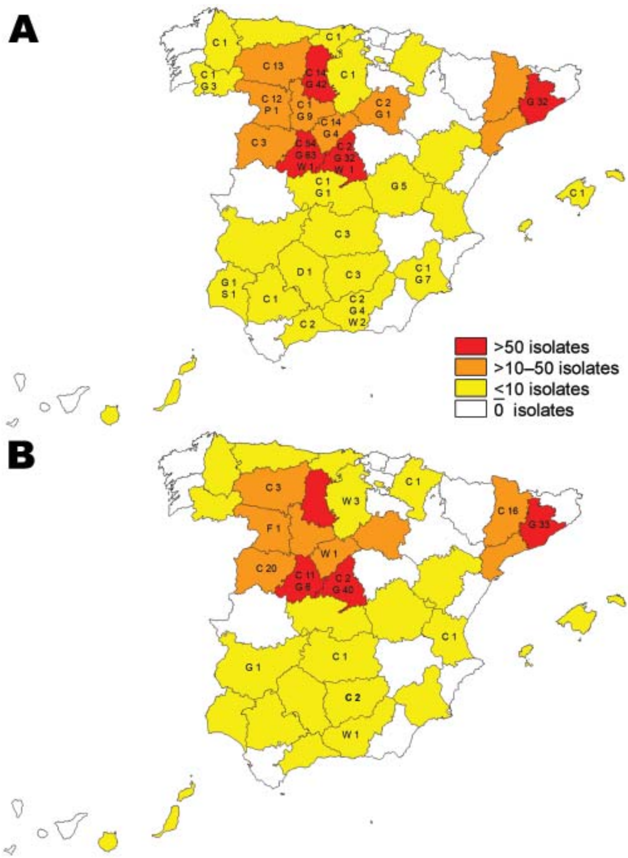

Figure 1. Map of Spain showing the distribution of the 2 most frequent *Mycobacterium caprae* spoligotypes and affected animals: C, cattle; D, red deer; F, fox; G, goats; S, sheep; P, pigs; WB, wild boar. A) Spoligotype SB0157. B) Spoligotype SB0416.

Author affiliations: Universidad Complutense de Madrid, Madrid, Spain (S. Rodríguez, J. Bezos, B. Romero, L. de Juan, J. Álvarez, E. Castellanos, N. Moya, F. Lozano, A. Mateos, L. Domínguez, A. Aranaz); University of Agriculture, Faisalabad, Pakistan (M.T. Javed); Ministerio de Medio Ambiente, y Medio Rural y Marino, Madrid, (J.L. Sáez-Llorente); and European Food Safety Authority, Parma, Italy (E. Liébana)

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[1]A list of members of The Spanish Network on Surveillance and Monitoring of Animal Tuberculosis can be found in the online Technical Appendix (www.cdc.gov/EID/content/17/3/532-Techapp.pdf).

patterns were obtained from the *Mycobacterium bovis* Spoligotype Database (www.mbovis.org).

Further authentication was achieved by detection of RD4 in the isolates with a 3-primer PCR in a panel of 63 unrelated isolates that included all spoligotyping patterns and animal species. Of the selected isolates, 62 showed the 545-bp product, indicating that they harbor RD4. One isolate from a cow of Eastern European origin repeatedly showed a 340-bp band, and its sequencing could not confirm presence or absence of RD4. For detection of specific *M. caprae* gene polymorphisms, 1 isolate from every spoligotyping pattern was studied. Additional identification was determined by sequencing of the pyrazinamidase A gene, which demonstrated a C at nt 169 that results in the functional wild-type pyrazinamidase A gene, and of the gyrase B gene that showed the G at nt 1311 and a C at position 1410 (online Technical Appendix).

The isolates, which originated from 195 single cases or outbreaks (Table 1), clustered into 15 patterns, which share the features previously described for the species (absence of spacers 1, 3–16, 28, and 39–43). Notably, the Iberian spoligotype cluster lacks spacers 30–33, whereas most *M. caprae* isolates from central Europe belong to spoligotypes that harbor these spacers. The 3 isolates of profiles SB0418 and SB1619 that presented spacers 30–33 originated from cattle imported from southeastern Europe. The 2 predominant spoligotypes, SB0157 and SB0416, were found to be responsible for 60% and 22%, respectively, of the cases and infected different animal species in distant areas, whereas 7 patterns were unique to a single case or outbreak. We calculated the index of discrimination (D) described by Hunter and Gaston (*10*) using the website of the University of the Basque Country (www.insilico.ehu.es). The result, D = 0.584, is notably lower compared with a parallel research of 252 patterns from 6,215 *M. bovis* isolates (D = 0.87) (*11*).

Additionally, variable number tandem repeat typing by using loci ETR-A, ETR-B, ETR-D, QUB11a, QUB11b, QUB3232, ETR-E, and MIRU26 (online Technical Appendix) was performed as described by Frothingham and Meeker-O'Connell (*12*) on a selection of 20 isolates (Table 2). The isolates originated from 10 properties (6 goat herds and 4 cattle farms), each with 2 different spoligotypes detected at a time. At 5 farms, the loss of spacers 25–27, 29, and 34–38, which can be explained by a single deletion event, had caused a change of the spoligotype pattern. This loss changed SB0157 to SB1081 and SB1084 to SB1889, while the variable number tandem repeat profiles within the same farm remained identical.

The routine application of molecular diagnosis and typing techniques in clinical laboratories has enabled its real role as a pathogen for several species to be recognized. In Spain, *M. caprae* represents 7.4% of all *M. tuberculosis* complex isolates from domestic and wild animals. Seventy-five of the 197 outbreaks (38.1%) involved goats (Table 1). This species showed the highest diversity among *M. caprae* with 12 patterns identified, 6 of them exclusive to caprine herds. The association of *M. caprae* with goats in Spain may be due to 2 reasons. First, the microorganism seems to be highly pathogenic for the goats in Spain, based on the

Table 1. Spoligotyping results of 791 *Mycobacterium caprae* isolates and their distribution within different animal species, Spain, 1992–2009*

Ref	Spoligotyping pattern†	No. animals (no. outbreaks)						
		Goats	Sheep	Cattle	Pigs	Wild boar	Red deer	Fox
SB0157	□■□□□□□□□□□□□□□□■■■■■□□■■■■□■□□□□■■■■■□□□□□	204 (44)	1	133 (67)	1	4 (4)	1	
SB0415	□■□□□□□□□□□□□□□□■■□■■■■■■■■□■□□□□■■■■■□□□□□	37 (4)	1	8 (4)		4 (3)		
SB0416	□■□□□□□□□□□□□□□□■■■■■■■■■■■□■□□□□■■■■■□□□□□	80 (18)		58 (21)		5 (3)		1
SB0866	□■□□□□□□□□□□□□□□■■■■■■■■□□□□□□□□□□□□□□□□□□□	1		1	1			
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SB1078	□■□□□□□□□□□□□□□□■■■■□□□■■■■□■□□□□■■■■■□□□□□	1		1				
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SB1081	□■□□□□□□□□□□□□□□■■■■■□□■□□□□□□□□□□□□□□□□□□□	1		8 (4)				
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SB1690	□■□□□□□□□□□□□□□□■■■■■■□■■■■□■■■■■■■■■■□□□□□			1				
Total		542 (75)	2 (2)	229 (105)	2 (2)	14 (11)	1	1

*Ref, reference. Numbering according to www.Mbovis.org.
†■, presence of spacer ; □, absence of spacer.

Table 2. Variable number tandem repeat analysis of isolates from 10 farms that presented mixed *Mycobacterium caprae* infection (different spoligotype patterns), Spain, 1992–2009*

			No. alleles at locus							
Farm	Animal	Spoligotype	ETR-A	ETR-B	ETR-D	QUB3232	QUB 11a	QUB 11b	MIRU 26	MIRU 31
1	Goat	SB0416	4	4	4	8	7	2	5	2
		SB0866	5	3	3	8	7	4	2	4
2	Goat	SB0416	4	3	4	8	7	2	4	2
		SB0157	4	3	4	8	7	2	4	2
3	Goat	SB0416	4	5	5	7	6	4	5	5
		SB0415	5	1	3	8	7	3	5	5
4	Cattle	SB0157	3	3	4	8	7	2	5	2
		SB1081	3	3	4	8	7	2	5	2
5	Cattle	SB0157	4	3	4	3	7	2	5	2
		SB1081	4	3	4	3	7	2	5	2
6	Goat	SB0157	4	3	4	8	7	2	5	2
		SB1078	4	3	4	8	7	2	5	2
7	Goat	SB1084	5	1	3	9	5†	3	5	4
		SB1889	5	1	3	9	5†	3	5	4
8	Cattle	SB0157	4	3	4	8	7	2	5	2
		SB1081	4	3	4	8	7	2	5	2
9	Cattle	SB0416	5	3	3	8	6	4	2	3
		SB0157	4	3	4	8	7	2	5	2
10	Goat	SB0973	4	3	–	–	–	–	–	–
		SB0157	4	3	4	9	–	2	5	–

*–, no amplification.
†Gel band of ≈1,800 bp. Sequencing showed that insertion sequence IS*6110* is inserted within the third repetition of QUB11a.

disseminated tuberculous lesions that it produces and its fast transmission within a herd. Second, caprine herds have not been included in the national eradication campaign (except when coexisting with cattle or as part of some regional programs). Therefore, *M. caprae* infection can spread easily through animal movements, such as purchase for replacement or genetic improvement.

The emergence of this pathogen in cattle has been observed. Cattle were involved in 106 outbreaks (53.3%) during the study period. Since 2004, cattle from 2,218 herds identified in the eradication program have been inspected by bacteriology. The number of cattle properties infected with *M. caprae* represented 0.85%–6.67% of the total number of herds diagnosed with bovine tuberculosis. Temporal trend of *M. caprae* isolates cultured over time was assessed by using the software WINPEPI 9.4 (*13*). The proportion of *M. caprae* isolated from bovine samples has increased consistently during 2004–2009, showing a significant positive trend ($p = 0.009$, by Mantel trend test) (Figure 2). We observed more *M. caprae* infections in cattle in regions with a high goat density. However, an analysis of the type of farm production shows that 86.7% of *M. caprae*–infected cattle have been raised in farms without any contact with small ruminants. This fact indicates recirculation of the pathogen within and between cattle herds. In countries that are virtually free of animal tuberculosis such as Germany, Austria, and the Czech Republic, a large number of cases in cattle and red deer are caused by *M. caprae*.

Identification of isolates from human patients has shown *M. caprae* as a human pathogen (*3*,*6*,*14*). A recent study suggests that *M. caprae* causes 0.3% of the cases of human tuberculosis in Spain, with SB0157 also being the most dominant spoligotype (*14*). The role of the pathogen as a public health risk is highlighted by lesions that can

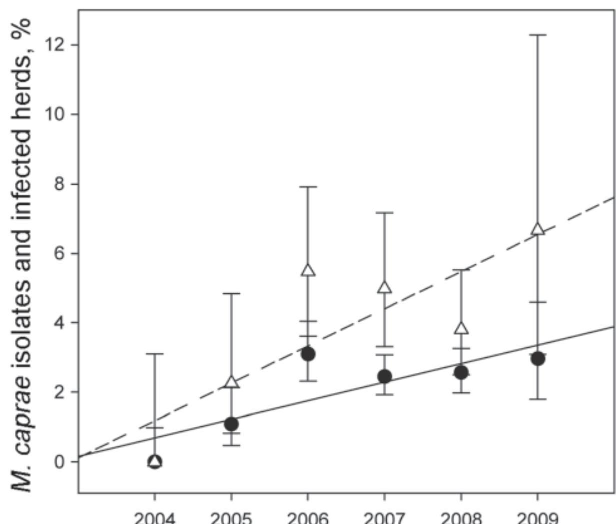

Figure 2. Proportion and regression lines of *Mycobacterium caprae* isolates (black dots, continuous line) and *M. caprae*–infected herds (white triangles, dashed lines) of the total number of *M. tuberculosis* complex isolates and *M. tuberculosis* complex–infected herds identified in cattle during 2004–2009. Error bars indicate 95% confidence intervals.

also be found in the mammary glands of infected goats; thus, consumption of unpasteurized dairy products remains a concern (*15*).

Conclusions

Compelling evidence indicates that *M. caprae* poses a serious health risk not only for goats, but also for other domestic and wild animal species and humans. Our results indicate that *M. caprae* infection is widespread in Spain and that the epidemiology is driven by caprine infections. Considering the role of *M. caprae* in animal tuberculosis, relevant legislation should be considered to address the infection as was done for *M. bovis*.

Acknowledgments

We thank the National and Regional Animal Health authorities, especially L. Carbajo, for their continuous encouragement. We are grateful to T. Alende, A. Gutiérrez, C. Viñolo, L. Guijarro, J. Gimeno, N. Álvarez, N. Montero, C. Lozano, L. Pazos and S. González for technical help. We acknowledge the staff of SADNA (Centro de Investigaciones Biológicas, Madrid) for sequencing.

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Ms Rodríguez is a PhD candidate in the Departamento de Sandidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid and received predoctoral research fellowship AP2006-01630 from the Spanish Ministry of Education. Her research focuses on molecular characterization of *M. tuberculosis* complex isolates and its application in epidemiology of these pathogens.

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Sporadic Human Cryptosporidiosis Caused by *Cryptosporidium cuniculus*, United Kingdom, 2007–2008

Rachel M. Chalmers, Kristin Elwin, Stephen J. Hadfield, and Guy Robinson

To investigate sporadic human cryptosporidiosis trends in the United Kingdom, we tested 3,030 *Cryptosporidium* spp.–positive fecal samples, submitted for routine typing in 2007–2008, for *C. cuniculus. C. cuniculus* prevalence was 1.2%; cases were mostly indigenous and occurred across all age groups. Most occurred during August–October and may be linked to exposure opportunities.

The protozoan parasites *Cryptosporidium* spp. are major causes of gastrointestinal disease worldwide. Most cases in the United Kingdom are caused by *C. parvum* or *C. hominis*; rare infections with other species and genotypes include *C. meleagridis*, *C. felis*, *C. canis*, *C. ubiquitum*, *C. hominis* monkey, skunk, horse, and rabbit (*1–3*). In summer 2008, the rabbit genotype caused a waterborne outbreak in drinking water (*4*). Previously, only 1 human infection, also identified in the United Kingdom, was known (*1*), although routine typing based on *RsaI* restriction fragment-length polymorphisms (RFLPs) within the *Cryptosporidium* oocyst wall protein (COWP) gene (*2*) does not differentiate the rabbit genotype from *C. hominis* (*4*). After phylogenetic and biologic investigations, the rabbit genotype has been renamed *C. cuniculus* (*5*). However, information is lacking about the occurrence and epidemiology of animal and human infections outside the outbreak, which mainly involved adult females (*5–7*). To investigate trends in humans, we conducted enhanced testing of *Cryptosporidium* spp.–positive fecal samples from patients with sporadic diarrhea (submitted for routine typing during 2007–2008) with the purpose of identifying and characterizing *C. cuniculus*.

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The Study

Archived DNA from all samples received for typing during January 2007–December 2008 that showed *C. hominis* COWP PCR-RFLP *RsaI* profiles were retested by single-round small subunit (SSU) rRNA PCR-RFLP using *SspI*, which generates a pattern unique to *C. cuniculus* (*4*). An exception occurred during the outbreak period (July and August 2008) when all *Cryptosporidium* spp.–positive stool samples were tested by a pan-genus nested PCR specific for the SSU rRNA gene and products digested with *SspI* and *VspI* (*4*). Although differentiating more species/genotypes than the COWP PCR-RFLP (*7*), this assay is unsustainable for typing large numbers of samples on a routine basis. *C. cuniculus* was confirmed by sequencing ≈830 bp of the SSU rRNA gene and ≈850 bp of the 60-kDa glycoprotein (GP60) gene by using nested PCR protocols (*4*).

Data were analyzed in Epi Info version 6 (Centers for Disease Control and Prevention Atlanta, GA, USA). Incomplete data for Northern Ireland were excluded, but no *C. cuniculus* cases were identified there. Case-patients with sporadic *C. cuniculus*, *C. parvum*, and *C. hominis* infections were compared by age using the Mann-Whitney 2-sample test, by sex using the Mantel-Haenszel version of the χ^2 test, by month of specimen submission, and by Government Office Region (England and Wales) or Health Board (Scotland) of the primary diagnostic laboratory.

In total, 37 (1.2%) of 3,030 infections were caused by *C. cuniculus*: 23 in 2007 and 14 in 2008 (online Appendix Table, www.cdc.gov/EID/content/17/3/536-appT.htm). Twenty-five were in patients from England and Wales, and 12 patients were from Scotland. Other cryptosporidia detected were *C. parvum* (n = 1,506, 49.7%), *C. hominis* (n = 1,383, 45.6%), *C. meleagridis* (n = 26), *C. felis* (n = 8), cervine genotype (n = 8), co-infection *C. hominis* and *C. parvum* (n = 5), novel or unidentified genotypes (n = 5), and *C. hominis* monkey genotype (n = 1); 88 did not amplify with the PCR primers. Substitution of routine typing with the SSU rRNA nested PCR-RFLP during the outbreak did not increase the number of "unusual" cryptosporidia, apart from *C. cuniculus*, indicating the routine COWP PCR-RFLP is otherwise appropriate for typing for epidemiologic purposes in the United Kingdom.

The age range of patients with sporadic *C. cuniculus* infection was 1–74 years (mean 29 years; median 31 years), significantly older than *C. hominis* case-patients (range 0–83 years; mean 19 years; median 13 years) (Mann-Whitney 2-sample test value = 11.12, df = 1, p = 0.0009) and *C. parvum* case-patients (range 0–86 years; mean 17 years; median 29 years) (Mann-Whitney 2-sample test value = 15.24, df = 1, p = 0.00009) (Figure 1). The sex distribution was 14 (37%) female and 22 (58%) male patients, with the sex of 1 patient not known, compared with sporadic *C. parvum* (781 [51.9%] female) and *C. hominis*

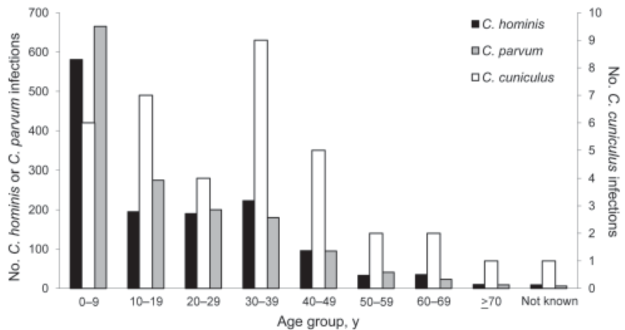

Figure 1. Age distribution of patients with sporadic cases of *Cryptosporidium cuniculus*, *C. hominis*, and *C. parvum* infection in England, Wales, and Scotland, 2007–2008.

(736 [53.2%] female) cases, although the difference was not significant ($\chi^2 = 4.01$, df = 2, p = 0.13).

More *C. cuniculus* cases were detected in the late summer and autumn than in the winter and spring, similar to infections with *C. hominis* but not *C. parvum* (Figure 2). Three *C. cuniculus* cases were identified in the East Midlands, the outbreak affected region, but none were found in Northamptonshire, the outbreak-affected area. Most cases (24%) were in the Eastern region of England. Two case-patients had traveled outside the UK, one to Spain and the other destination not known, during the incubation period.

Occupational and environmental exposure data were available for 14 *C. cuniculus* case-patients from patient questionnaires administered by local Environmental Health Departments. No occupational risks were linked to rabbits. One patient (a 9-year-old boy) reported direct contact with rabbits (a pet), and 2 patients had potential environmental contact (a 63-year-old woman played golf, and a 36-year-old man sat on grass during a walking holiday). Two case-patients reported diarrhea in other residential contacts. None of the patients reported links with (lived in, had visited or received visitors from) the outbreak-affected area. Available clinical details were insufficient for all cryptosporidiosis case-patients for comparative purposes. One *C. cuniculus* case-patient was an immunosuppressed child who had received a kidney transplant.

Two GP60 subtype families, Va (n = 18) and Vb (n = 19), were detected in sporadic *C. cuniculus* isolates, linked to patient sex; 10/14 (71.4%) female patients had Va subtype, compared with 7/22 (31.8%) male patients ($\chi^2 = 5.24$, df = 1, p = 0.022). No significant difference in age or regional distribution was found, but cases with Va occurred only in August through December, while Vb cases occurred all year but mostly in August (Figure 2).

Representative sequences have been deposited into GenBank: GU971631–GU971650 (GP60) and GU971628–GU971630 (SSU DNA). The latter are identical to those deposited previously (EU437413, FJ262724–FJ262726) (*1*,*4*) and are *C. cuniculus* (*5*).

Conclusions

C. cuniculus was first identified as a human pathogen during a waterborne outbreak, and its epidemiology has now been described for sporadic cases in the United Kingdom. Although the numbers are small, and the data need to be interpreted with caution, it was the third most commonly identified *Cryptosporidium* species in patients with diarrhea during the study period, after *C. parvum* and *C. hominis*. All *C. cuniculus* isolates identified by PCR-RFLP were confirmed by sequence analysis, indicating the reliability of the test algorithm used, although the development of specific probes will enhance testing capability. All ages were infected with little age delimitation <50 years, after which numbers declined. Contrast this to *C. parvum* and *C. hominis*, which are both linked to young age. *C. cuniculus* distribution is seasonal, peaking in August through November, and differences in seasonal distribution of GP60 subtypes were marked.

Rabbits are the natural hosts for *C. cuniculus* (*5*), and seasonal distribution and variation in humans may reflect rabbit breeding seasons and infections, although good epidemiologic studies of *Cryptosporidium* spp. in wild rabbits are lacking (*7*). Studies of farmed and wild rabbits so far indicate that GP60 subtype Vb predominates (*7*–*9*), although the outbreak was caused by Va (*4*). Unlike with *C. hominis*, seasonal distribution of cases was not linked to foreign travel. The *C. cuniculus* outbreak had occurred in July when few sporadic cases were detected. The distribution of sporadic cases was the opposite of the outbreak in which more case-patients were female (*6*). The association between GP60 subtypes and patient sex is intriguing. A small number of case-patients had possible exposure risks, although this requires further

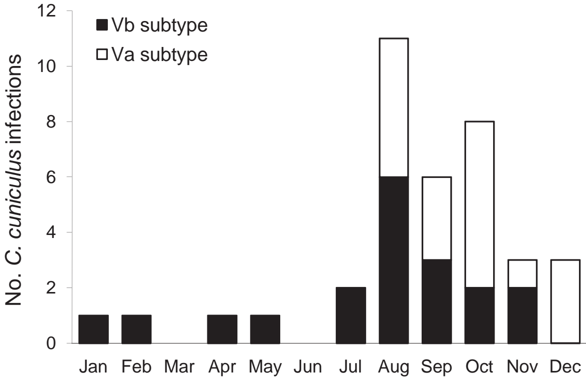

Figure 2. Monthly distribution of sporadic cases of *Cryptosporidium cuniculus*, *C. hominis*, and *C. parvum* infection in England, Wales, and Scotland, 2007–2008.

investigation. The only known hosts of *C. cuniculus* are humans and European rabbits (*Oryctolagus cuniculus*) (*5*,*7*–*9*), and until population-based studies of rabbits are undertaken, the risks cannot be fully evaluated, nor the human epidemiology fully explained.

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Dr Chalmers is head of the UK Cryptosporidium Reference Unit, Public Health Wales. Her main research interests are the epidemiology, management, and control of *Cryptosporidium* spp. and cryptosporidiosis.

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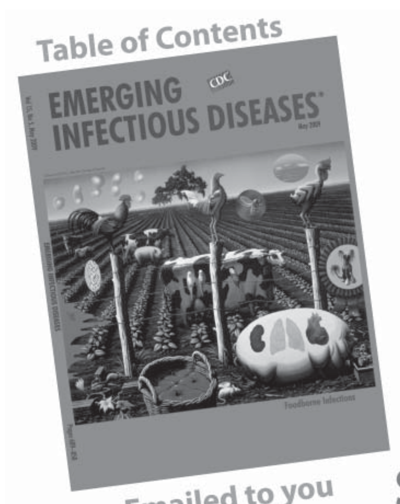

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Isoniazid-Resistant Tuberculous Meningitis, United States, 1993–2005

Christopher Vinnard, Carla A. Winston, E. Paul Wileyto, Rob Roy MacGregor, and Gregory P. Bisson

To determine patient characteristics associated with isoniazid resistance in cases of tuberculous meningitis, we conducted a cross-sectional study by using data from the US National Tuberculosis Surveillance System during 1993–2005. Foreign-born patients were more likely to be infected with an isoniazid-resistant strain.

The mortality rate for tuberculous meningitis (TBM) is higher than other forms of tuberculosis (TB), and survivors are often left with severe neurologic disability (*1*). We have recently shown that infection with isoniazid-resistant (rifampin-susceptible) *Mycobacterium tuberculosis* was associated with a 2-fold increase in the odds of death during therapy among patients with TBM who had positive cerebrospinal fluid (CSF) cultures, compared with patients with isoniazid-susceptible cases (*2*). When patients have a history of TB, clinicians may consider treatment history and drug susceptibilities in choosing empiric therapy (*3*). In contrast, little guidance is available to the clinician in the selection of an empiric regimen for patients without a history of treatment. Given that host and pathogen genotypes have been found to jointly influence the propensity of *M. tuberculosis* to cause meningeal infection, the epidemiology of isoniazid-resistant TBM may be different for meningeal and nonmeningeal forms of TB (*4*). We sought to determine the patient characteristics independently associated with isoniazid resistance on initial susceptibility testing among patients with TBM in the United States.

The Study

We performed a cross-sectional study of clinical and demographic factors associated with isoniazid resistance on initial susceptibility testing in patients with TBM by using data from the United States National Tuberculosis Surveillance System. We examined data on all TB cases reported from January 1, 1993, through December 31, 2005. Patients were included if a clinical diagnosis of meningitis was made, positive cultures for *M. tuberculosis* were obtained from CSF, and results of any initial drug susceptibility testing were recorded. To study factors associated with isoniazid resistance in patients without a treatment history, we excluded patients with a previous diagnosis of TB. We also excluded patients with multidrug-resistant disease on the basis of evidence for differences in the epidemiology of isoniazid-resistant (rifampin-susceptible) and multidrug-resistant TB (*5*).

Differences in characteristics between the isoniazid-resistant and isoniazid-susceptible groups were assessed by using χ^2 test and were selected for evaluation in a multivariable logistic regression model if unadjusted analysis demonstrated an association ($p<0.25$). An odds ratio (OR) for the association between a patient characteristic and initial isoniazid resistance was determined, along with its associated 95% confidence interval (CI). Multiple imputation was used to account for missing observations and permit complete data methods for analysis, under the assumption that missing data followed a missing-at-random pattern (*6*). Likelihood ratio testing was used to compare nested models, and the Akaike Information Criteria were used to compare non-nested models.

During 1993–2005, a total of 1,649 patients had a diagnosis of TBM, no previous history of TB, positive CSF cultures, and initial drug susceptibility testing. Of these 1,649 patients, 234 patients (14%) were infected with an isolate resistant to at least 1 first-line agent (isoniazid, rifampin, ethambutol, pyrazinamide, or streptomycin). Overall, 133 of 1,649 (8%) patients were infected with an isolate resistant to at least isoniazid.

After we excluded 11 patients without susceptibility testing results for isoniazid and 24 patients with multidrug-resistant disease, we compared 109 patients with at least isoniazid-resistant disease with 1,505 patients with isoniazid-susceptible disease. Unadjusted associations of clinical and demographic characteristics with initial isoniazid resistance are shown in Table 1. Foreign-born patients were more likely than US-born patients to have isoniazid-resistant disease, with an OR of 2.53 (95% CI 1.66–3.88). Overall, 849 of 1,614 (53%) patients in the primary analysis had a known HIV status, and 765 of 1,614 (47%) patients had unknown HIV status. Of the patients with known HIV status, 362 of 849 (43%) were HIV positive, and 487 of 849 were HIV negative (57%). HIV infection was not associated with initial isoniazid resistance (OR 1.10, 95% CI 0.62–1.95). Among HIV-positive patients, the association between foreign birth and initial isoniazid resistance was 3.05 (95% CI 1.54–6.06), and among HIV-negative patients it was 1.60 (95% CI 0.70–3.65).

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Based on unadjusted analyses, the multivariable model included age, race, residence in a long-term care facility, and foreign birth. Only foreign birth remained independently associated with initial isoniazid resistance (Table 2). Before age was adjusted for, the OR for foreign birth and initial isoniazid resistance was 2.53 (95% CI 1.66–3.88), and after adjusting for age, the OR was 2.25 (95% CI 1.47–3.43). Mexico was the most commonly reported country of origin

Table 1. Unadjusted analysis of factors associated with initial isoniazid resistance in tuberculosis disease, United States, 1993–2005*

Patient characteristics	No. cases with isoniazid resistance/total no. cases (%)	OR (95% CI)	p value
Origin†			
US-born	40/926 (4)	Reference	
Foreign-born	69/674 (10)	2.53 (1.66–3.88)	<0.01
Age category, y			<0.01
<1	1/57 (2)	0.14 (0–0.86)	
1–<4	4/94 (4)	0.34 (0.08–1.00)	
4–<14	3/50 (6)	0.49 (0.09–1.67)	
14–<24	8/111 (7)	0.59 (0.23–1.38)	
24–<34	31/268 (12)	Reference	
34–<44	29/344 (8)	0.70 (0.40–1.24)	
44–<54	14/247 (6)	0.46 (0.22–0.92)	
54–<64	11/157 (7)	0.58 (0.25–1.22)	
64–74	5/159 (3)	0.25 (0.07–0.66)	
>74	3/127 (2)	0.18 (0.04–0.61)	
Race category			
White, non-Hispanic	14/240 (6)	Reference	0.03
Black, non-Hispanic	27/578 (5)	0.79 (0.39–1.66)	
Hispanic	38/489 (8)	1.36 (0.70–2.78)	
Asian/Native Hawaiian, non-Hispanic	28/276 (10)	1.82 (0.90–3.84)	
American Indian, non-Hispanic	0/16	0 (0–4.02)	
HIV status‡			
Negative	32/487 (7)	Reference	
Positive	26/362 (7)	1.10 (0.62–1.95)	0.73
Sex			
F	48/650 (7)	Reference	
M	61/964 (6)	0.85 (0.56–1.28)	0.41
Homeless within the previous year			
No	99/1,426 (7)	Reference	
Yes	3/74 (4)	0.57 (0.11–1.78)	0.48
Resident of a long-term care facility at diagnosis			
No	105/1,491 (7)	Reference	
Yes	2/67 (3)	0.41 (0.05–1.57)	0.20
Resident of a correctional facility at diagnosis			
No	106/1,561 (7)	Reference	
Yes	3/43 (7)	1.03 (0.20–3.32)	0.96
Pulmonary disease			
No	69/1,068 (6)	Reference	
Yes	40/546 (7)	1.14 (0.74–1.74)	0.51
Abnormal chest radiograph results			
No	48/690 (7)	Reference	
Yes	58/835 (7)	1.00 (0.66–1.52)	0.99
Positive smear (nonsputum site)			
No	71/985 (7)	Reference	
Yes	32/434 (7)	1.02 (0.64–1.61)	0.91
Positive tuberculin skin test result			
No	24/433 (5)	Reference	
Yes	36/520 (7)	1.33 (0.75–2.38)	0.30

*OR, odds ratio; CI, confidence interval.
†US-born persons were defined as persons born in the United States, Puerto Rico, or US outlying area, or born abroad to American parents; all other persons were defined as foreign-born.
‡California reported only patients matched to the California AIDS registry from 1993–2004 as HIV positive; all other California patients are missing HIV status.

Table 2. Adjusted analysis of factors associated with initial isoniazid resistance in tuberculosis disease, United States, 1993–2005*

Characteristic	Adjusted OR (95% CI)	p value
Foreign-born	2.25 (1.47–3.43)	<0.01
Age category, y		0.10
<1	0.22 (0.03–1.70)	
1–<4	0.52 (0.17–1.57)	
4–<14	0.60 (0.17–2.06)	
14 –<24	0.53 (0.24–1.21)	
24–<34	Reference	
34– <44	0.80 (0.46–1.37)	
44–<54	0.54 (0.28–1.06)	
54 <64	0.60 (0.29–1.24)	
64–74	0.28 (0.11–0.74)	
>74	0.22 (0.07–0.74)	

*OR, odds ratio; CI, confidence interval.

for foreign-born patients, accounting for 20 of 69 foreign-born patients with isoniazid-resistant disease. Countries in Asia accounted for 7 of 9 countries with ≥2 cases of isoniazid-resistant TBM (Figure).

Conclusions

In this national cohort of patients with TBM, initial isoniazid resistance was more commonly seen in patients born outside the United States. In an earlier study of all forms of isoniazid monoresistant TB in the United States, foreign birth was also found to be a significant risk factor (*5*). Although we excluded patients with a diagnosis of TB, clinicians may have been unaware of a patient's previous episode of TB that was treated before their arrival in the United States. Foreign-born patients may also have emigrated from countries with a higher prevalence of isoniazid resistance among newly diagnosed cases. Initial isoniazid resistance was also uncommon in persons >64 years of age. Older persons may have been exposed to TB antecedent to the use of isoniazid in treatment regimens, leading to reactivation with a drug-susceptible strain.

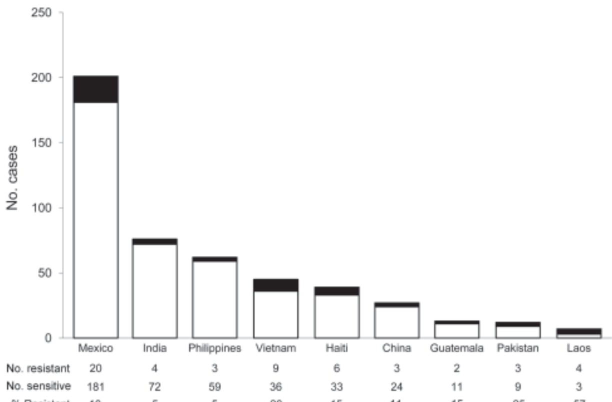

Figure. Countries of origin for foreign-born persons with tuberculous meningitis, United States, 1993–2005. Black bar sections indicate isoniazid-resistant and white bar sections indicate isoniazid-sensitive tuberculosis.

HIV infection is associated not only with increased risk for progression to active TB, but also an increased risk for extrapulmonary involvement among patients with active cases, including an increased risk for TBM (*7*). However, we did not see an association between HIV and initial isoniazid resistance among persons with known HIV status (p = 0.73), and the strength of the association between foreign birth and initial isoniazid resistance was not significantly modified by the presence of HIV infection. Similar to our findings, a lack of association between HIV and isoniazid resistance was seen in all cases of TB in the United States (with 13% known HIV positive) and the United Kingdom (with 5% known HIV positive) (*5*,*8*).

HIV status was missing for 47% of patients with TBM during the study period. For all states except California, reporting of HIV status to the National Tuberculosis Surveillance System increased from 36% in 1993 to 79% in 2008 (*9*). California reported only patients matched to the California AIDS registry during 1993–2004 as HIV positive. All other California patients are missing HIV status.

This study had several other limitations. Individual MIC levels for isoniazid were unavailable, and reporting does not distinguish between low-level and high-level resistance. Although we excluded patients with a history of TB, we were unable to identify patients previously treated for latent TB, which was shown to be associated with isoniazid monoresistance in patients for whom active TB subsequently developed (*10*). In conclusion, foreign-born persons with TBM who seek care in the United States were more likely to be infected with an isoniazid-resistant strain of *M. tuberculosis* compared with US-born persons, and persons >64 years of age were less likely to have an isoniazid-resistant infection than were persons 25–34 years of age. Prospective studies are needed to determine whether individual patient characteristics can guide the selection of TBM therapies and lead to an improvement in clinical outcomes.

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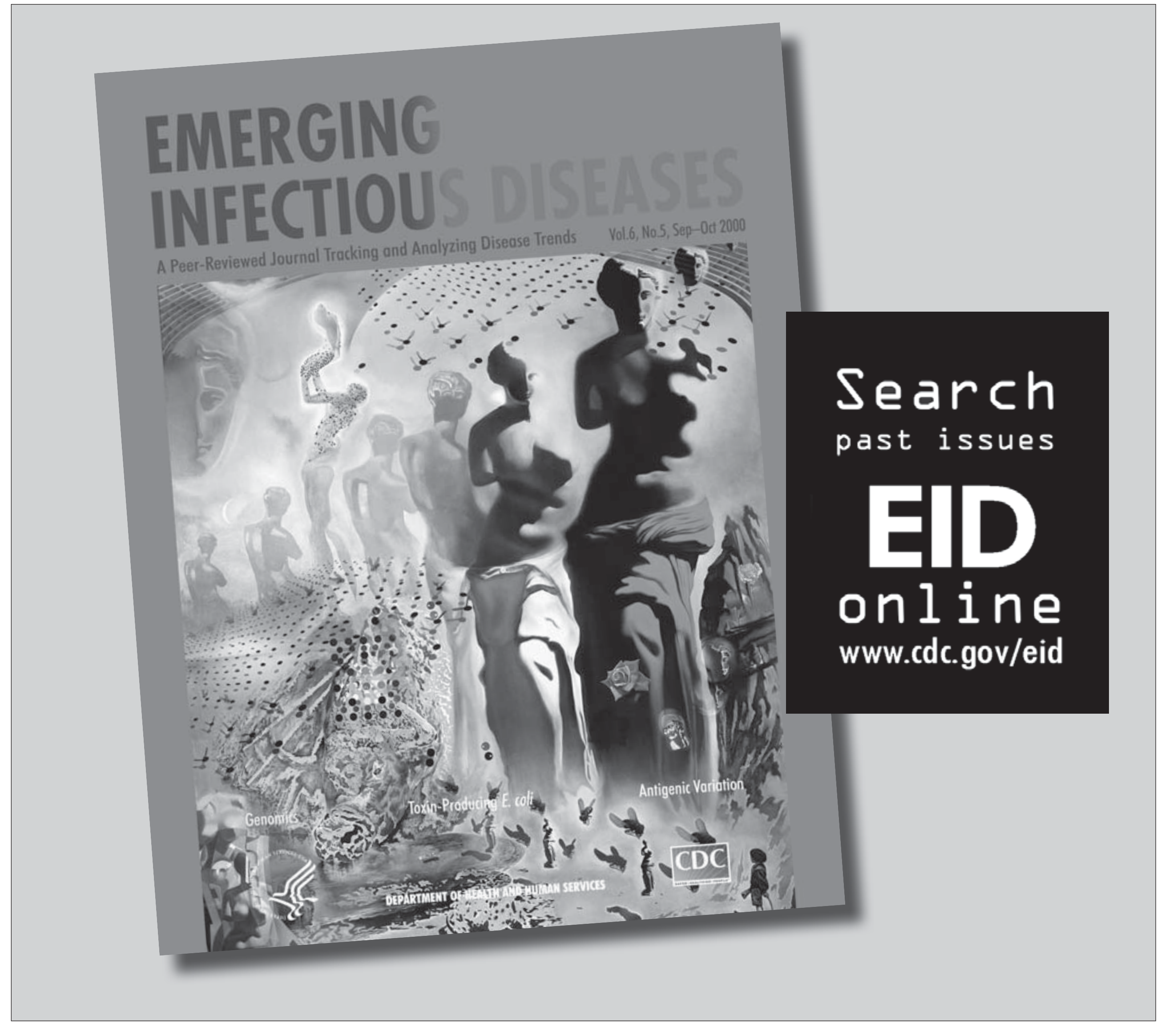

Surveillance for Invasive Meningococcal Disease in Children, US–Mexico Border, 2005–2008[1]

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We reviewed confirmed cases of pediatric invasive meningococcal disease in Tijuana, Mexico, and San Diego County, California, USA, during 2005–2008. The overall incidence and fatality rate observed in Tijuana were similar to those found in the US, and serogroup distribution suggests that most cases in Tijuana are vaccine preventable.

Invasive meningococcal disease (IMD) is caused by *Neisseria meningitidis*. Specific antibodies against the capsule are used to define the 13 known *N. meningitidis* serogroups (*1*). In the United States, *N. meningitidis* is a leading cause of bacterial meningitis (*2*,*3*). According to the provisional Active Bacterial Core Surveillance report of the Centers for Disease Control and Prevention, 1,050 cases of IMD were estimated to occur in 2008, with an overall incidence of 0.33/100,000 population and mortality rate of 0.03/100,000 population. Higher age-specific incidence and proportion of deaths occur in children and adolescents (*4*). In the United States, Active Bacterial Core Surveillance data show that serogroups B (0.11/100,000), C (0.11/100,000), and Y (0.08/100,000) are predominant (*5*).

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IMD is a reportable condition in both the United States and Mexico. In 2006, the Mexican National Epidemiologic Surveillance System reported 60 cases in Mexico (population 105,790,700) for a nationwide rate of 0.056/100,000 (*6*). However, only a limited number of epidemiologic descriptions of IMD, primarily from outbreaks, are available from Mexico. For example, an outbreak of 753 cases was recorded during 1945–1949 in San Luis Potosi. Most cases were among infants and young children; serogroup data were not available (*7*).

Although physicians in Mexico at the US–Mexico border areas often encounter patients with symptoms highly compatible with IMD, diagnosis is not routinely culture-confirmed; this likely leads to underreporting. Serogroup-specific data on IMD are also lacking elsewhere throughout Mexico. The goals of our study were to compare hospital-based estimates of IMD in children and serogroup distribution at Tijuana General Hospital (TGH), Mexico, with a catchment population of nearly 200,000 children <17 years, to reported IMD cases in children in San Diego County (SDC), with a population of 723,600 children <17 years. (All demographic and serogroup data are listed in the Table.) This border is the most traversed international frontier in the world. We hypothesized that rates of IMD are underreported at TGH and that serogroup distribution is similar on both sides of the US–Mexico border.

The Study

During October 1, 2005–May 31, 2008, active surveillance for IMD was initiated at TGH among children <17 years of age. Blood or cerebrospinal fluid specimens, or both, were collected from all patients with suspected sepsis, meningitis, or purpura fulminans. Data on all pediatric IMD reported to the SDC Health and Human Services Agency through electronic laboratory notification or from infection control practitioners were retrospectively analyzed during the same period. Inclusion criteria as follows: per Centers for Disease Control and Prevention guidelines (*8*), we included only confirmed cases with sterile-site (blood/cerebrospinal fluid) isolation of *N. meningitidis*. At the SDC Public Health Laboratory, *N. meningitidis* isolates were identified by using API NH (bioMérieux, La Bolme-les-Grottes, France) and serogrouped by standard slide agglutination methods. At TGH, isolates were serogrouped by standard latex well agglutination methods using Pastore*x* Meningitis kit (Alere Ltd, Stockport, UK). Six case-patients from TGH and 7 from SDC with purpura fulminans, disseminated intravascular coagulation, or both, lacked culture-proven *N. meningitidis* infection and were

[1]Preliminary results of this study were presented as a poster at the 14th Pan-American Congress on Infectious Diseases, Campos do Jordao, Brazil, April 25–28, 2009.

Table. Characteristics of pediatric case-patients who had invasive meningococcal disease, Tijuana, Mexico, and San Diego County, California, USA, October 1, 2005–May 31, 2008*

Characteristics	No. (%) patients	
	Tijuana, n = 16	San Diego County, n = 13
Age, y		
<1	2 (12.5)	6 (46.2)
1–4	7 (43.8)	4 (30.8)
5–11	4 (25.0)	2 (15.4)
12–16	3 (18.8)	1 (7.7)
Male sex	8 (50.0)	9 (69.2)
Race/ethnicity		
Non-Hispanic white	0	6 (46.2)
Hispanic	16 (100)	3 (23.1)
Non-Hispanic black	0	2 (15.4)
Asian	0	0
Pacific Islander	0	0
Other/unknown	0	2 (15.4)
Month of onset		
November–February	12 (75.0)	7 (53.8)
March–June	2 (25.0)	4 (30.8)
July–October	2 (25.0)	2 (15.4)
Serogroup		
A	0	0
B	2 (12.5)	8 (61.5)
C	10 (62.5)	2 (15.4)
Y	2 (12.5)	1 (7.7)
W135	0	0
Not typeable	0	0
Not typed	2 (12.5)	2 (15.4)
Case-fatality rate	3 (18.8)	2 (15.4)

*Tijuana cases from hospital-based estimates and serogroup distribution at Tijuana General Hospital. San Diego County cases taken from those reported to the San Diego County Health and Human Services Agency through electronic laboratory notification or from infection control practitioners.

excluded from analysis. Because patients ≥17 years are not hospitalized in the Department of Pediatrics at TGH, they were also excluded from the SDC data.

Clinical, microbiologic, and demographic data from TGH and SDC were analyzed by using STATA, Version 9.2 (StataCorp, LP, College Station, TX, USA). Pearson χ^2 and Fisher exact tests were performed; p values <0.05 were defined as significant.

During the study period, a total of 29 pediatric cases of IMD were diagnosed, 16 at TGH (an estimated 3.08 annual cases/100,000 children <17 years) and 13 in SDC (0.69 annual cases/100,000 children <17 years). Children <5 years accounted for most IMD cases at both sites: 9 cases at TGH, and 10 in SDC (p = 0.24) (Figure 1). Of the 29 case-patients, 11 children were 1–4 years of age, and 8 were infants <1 year of age. Children <1 year of age accounted for 2 cases at TGH and 6 cases in SDC (p<0.05), with most infections in SDC caused by serogroup B. A slight male predominance (55.2%) was observed on both sides of the border, and 65.5% were diagnosed during November–February.

Overall, serogroup C was most commonly identified among the 29 cases (41.4%), followed by B (34.5%) and Y (10.3%); another 13.8% of cases were not serogrouped (2 cases each at TGH and SDC). A significant difference in serogroup was observed by site: serogroup C was most commonly identified at TGH (62.5%), whereas serogroup B was most common in SDC (61.5%) (Figure 2) (p = 0.005).

Of the 29 IMD case-patients from TGH and SDC, 5 children died, including 3 from TGH and 2 in SDC. Four of those who died were <5 years of age, and 1 child was 15 years of age. One fatal infection was known to be potentially vaccine preventable (caused by serogroup C), but the organisms in the other 4 deaths were not serogrouped.

Conclusions

Documented reports of confirmed IMD in Mexico are rare (*6*), resulting in an assumption that incidence is extremely low. However, other infectious diseases, including tuberculosis, HIV/AIDS, and hepatitis A, B, and C, are common in this border region and often occur at higher rates than elsewhere in the United States (*9*) This surveillance project describes active hospital-based surveillance and serogroup distribution of IMD in children on both sides of the US–Mexico border. The age and serogroup distribution differed greatly between sites, with SDC demonstrating more infant cases and serogroup B, while TGH demonstrated more child and adolescent cases and serogroups C and Y.

This study suggests that rates of IMD at TGH, and presumably Tijuana and elsewhere in Mexico, may be substantially higher than reported. During the study period,

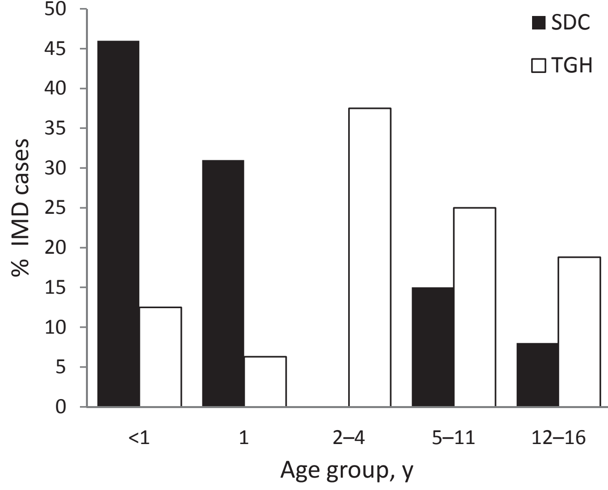

Figure 1. Cases of invasive meningococcal disease, by case-patient age group, Tijuana General Hospital (TGH), Tijuana, Mexico, and San Diego County (SDC), California, USA, October 1, 2005–May 31, 2008.

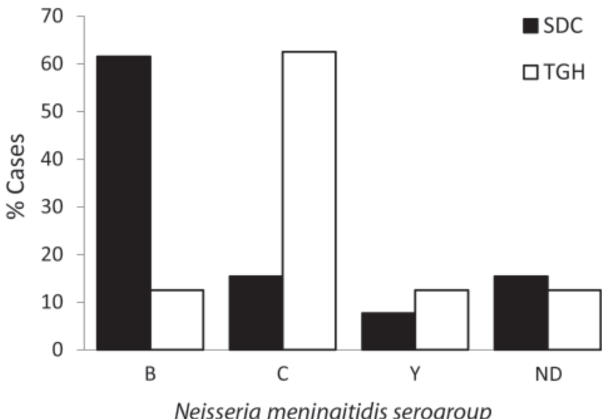

Figure 2. Cases of invasive meningococcal disease, by serogroup, Tijuana General Hospital (TGH), Tijuana, Mexico, and San Diego County (SDC), California, USA, October 1, 2005–May 31, 2008. ND, typing not done.

vaccine-preventable serogroups were more common in TGH than in SDC. This finding has potential implications for immunization with the meningococcal vaccines containing serogroups C and Y in Mexico. In the United States, the quadrivalent conjugated meningococcal vaccine is recommended for all persons 11–18 years of age and is indicated for persons 2–55 years of age who are at increased risk for IMD (*10*,*11*). This vaccine might have benefits in Tijuana in terms of carriage of the bacteria and reduction in serogroup-specific IMD incidence, effects which have been demonstrated elsewhere (*12*,*13*). Widespread meningococcal vaccination has not yet been introduced in Tijuana or elsewhere in Mexico, although the monovalent meningococcal C conjugate vaccine has been licensed in Mexico. This study suggests that a substantial number of IMD cases might have been prevented with quadrivalent conjugated meningococcal vaccine (75%) or monovalent serogroup C vaccine (63%). A recent study has shown that monovalent serogroup C vaccination administered to children <2 years of age could be effective in preventing IMD among infants (*14*).

This investigation was limited in several aspects, however. Tijuana serogroup data was only available for TGH (the city's indigent tertiary care referral center), which likely led to an underestimation of the number of pediatric IMD cases. Even though data were reviewed for nearly 3 years, the relatively small geographic area resulted in a small sample size, which limits generalizations.

IMD is likely to occur at a higher rate than previously reported in Tijuana. The overall incidence and fatality rate observed for TGH cases were similar to rates in the United States, and serogroup distribution at TGH indicates that most IMD cases in Tijuana are vaccine preventable. Establishment of a binational IMD surveillance system could provide substantial benefit in improving IMD control potentially leading to vaccination strategies in Mexico's northern border region, and perhaps elsewhere. Further IMD surveillance studies including binational systems are needed to better define the epidemiology of IMD in the northern border and other regions of Mexico and determine appropriate vaccination policies.

Dr Chacon-Cruz is a specialist in pediatric infectious diseases in the Department of Pediatrics and Infectious Diseases at Tijuana General Hospital, as well as head professor of pediatrics and professor of infectious diseases at the University of Xochicalco. His research interests include pediatric HIV and pneumococcal and meningincoccal diseases.

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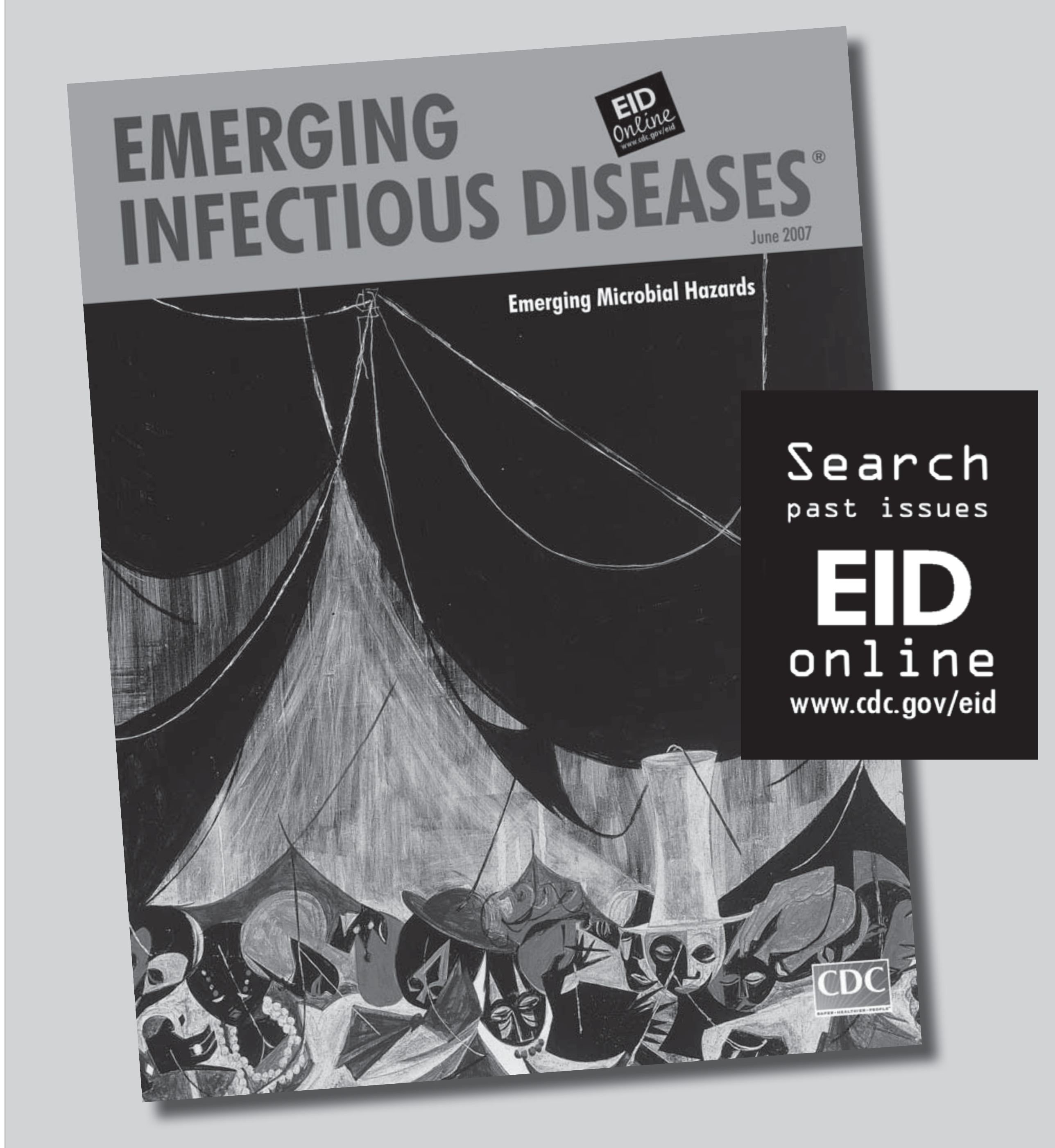
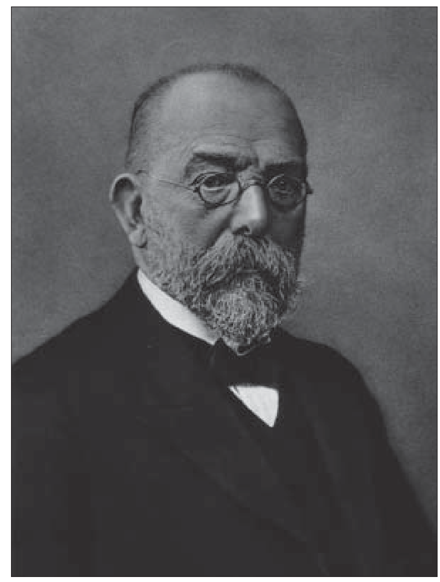


Who is this man?



Here is a clue: He discovered the organism that causes tuberculosis.

Who is he?

A) Paul Ehrlich

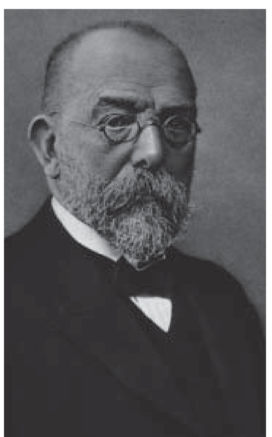
B) Robert Koch

C) Louis Pasteur

D) Edward Trudeau

E) Rudolf Virchow

Decide first. Then turn the page.



Robert Koch

Myron G. Schultz

This is a photograph of Heinrich Hermann Robert Koch (1843–1910). Koch in Germany and Louis Pasteur in France were the 2 main founders of the science of bacteriology. Koch is best known for his discovery of *Mycobacterium tuberculosis*, the organism that causes tuberculosis. For this discovery, Koch was awarded the Nobel Prize in Physiology or Medicine in 1905.

Koch was born in Clausthal, Prussia, the son of a mining official. He was 1 of 13 children, 2 of whom died in infancy. At 5 years of age, he astounded his parents by telling them that he had, with the aid of newspapers, taught himself how to read. Koch studied medicine at the University of Göttingen under the German anatomist and pathologist Friedrich Gustav Jakob Henle (1809–1885); he graduated in 1866. Koch was influenced by Henle, who believed that infectious diseases were caused by living, parasitic organisms. For a brief period, Koch studied in Berlin, where he came under the influence of Rudolf Virchow. He then served in the Franco–Prussian War and later became a district medical officer in Wollstein, Prussian Poland.

Koch's first major contribution, in 1876, was identification of the anthrax bacillus. Working as a provincial physician in the little town of Wollstein, far from other researchers, Koch used homemade equipment that he had invented to describe the entire life history of anthrax. He showed that the bacillus he found in blood samples could not survive for long outside a host but that it could exist for long periods under unfavorable circumstances in the form of spores. He originated a method of culturing the bacillus in the laboratory, outside the body of an infected animal.

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In 1880, Koch's work with anthrax earned him a position in the Imperial Health Office in Berlin. There he developed methods of isolating and cultivating disease-producing bacteria, and he formulated strategies for preventing their spread. In 1881, he published a report that advocated the need for pure culture for isolating disease-causing organisms and described in detail how to obtain them. His techniques for staining of bacilli, incubation, and pure culturing became the foundations of the science of bacteriology. He espoused the now-famous Koch's postulates, conditions that are essential for establishing that an organism is the cause of a disease. Koch's postulates state that an organism must be 1) found in all cases of the disease examined, 2) prepared and maintained in a pure culture, 3) capable of producing the original infection, even after several generations in culture, and 4) retrievable from an inoculated animal and cultured again.

In Berlin, Koch improved the methods that he had used while in Wollstein (e.g., staining and purification techniques) and bacterial growth media (e.g., agar plates and Petri dishes). With these techniques, Koch demonstrated the constant presence of the bacilli in the tuberculous lesions of men and animals; he cultivated these bacilli in pure culture on blood serum and produced tuberculosis at will by injecting the cultures into healthy animals. His findings were first presented in an article read before the Physiologic Society of Berlin on March 24, 1882. The logic, thoroughness, and importance of Koch's presentation deeply impressed his audience, and this date has become a landmark in medical history; March 24 is now celebrated annually as World Tuberculosis Day. In 1883, Koch published a detailed description of his research, which was later translated into English under the title, The Etiology of Tuberculosis.

In 1883, while still busy working on tuberculosis, Koch was sent to Egypt as leader of the German Cholera

Commission to investigate an outbreak of cholera. In Egypt, Koch discovered the vibrio organism that causes cholera and brought back pure cultures of it to Germany. He also studied cholera in India. Koch's work on cholera, for which he received an award of 100,000 German marks**,** had strong influence because he advocated regular checks of water supplies and sewage disposal. Other diseases that Koch investigated in Italy, South Africa, India, and Indonesia included malaria, black water fever (etiologically related to malaria), plague, rinderpest, and trypanosomiasis.

Koch faltered from his usual perfectionism when he announced at the Tenth International Medical Congress in Berlin, in 1890, that he had found an inoculum that could protect against tuberculosis and even cure the established disease. It was referred to for a while as "Koch lymph" and later as "old tuberculin." Koch's testimony provoked tremendous enthusiasm that a cure for tuberculosis had been found. Unfortunately, the healing powers that he claimed for tuberculin were greatly exaggerated. Evidence accumulated that patients often became sicker after tuberculin injection. Opinion turned against the use of tuberculin and against Koch. In later years, tuberculin became useful as a diagnostic agent in the tuberculin skin test.

Koch also faltered on the question of whether tuberculosis in cattle was caused by the same organism as tuberculosis in man. He believed that bovine tuberculosis was not a danger to humans. He espoused his beliefs at several conferences in the United States and Britain. In 1901, at the London Congress on Tuberculosis, he encountered strong disagreement from leading bacteriologists, including Joseph Lister, Edmond Nocard, Bernhard Bang, John McFadyean, and Theobald Smith. Koch's opinion had great influence because it was the testimony of an eminent authority. Yet, it was not backed by evidence. This modus operandi has led to many errors throughout medical history. In this instance, the consequences had enormous public health implications because this belief could have justified the use of tuberculous milk for human consumption. Within a few years, researchers, particularly Theobald Smith, and the English Royal Commission established beyond doubt that, contrary to Koch's belief, bovine tuberculosis was a serious problem in humans, especially children. The campaigns for the pasteurization of milk and eradication of bovine tuberculosis were the practical outcomes of evidence-based research.

Throughout history, tuberculosis has been described under many names. In the 19th century its name evolved from phthisis to consumption and then, after Koch's discovery, to tuberculosis. By whatever name, the amount of suffering brought about by tuberculosis is difficult to grasp. Wasting, fever, night sweats, breathlessness, pain in the side or shoulder, cough, abundance of sputum, and spitting of blood have long been associated with consumption. At the time of Koch's discovery, consumption was a common cause of illness and death in Europe. Industrialization caused overcrowding of cities, and inhabitants suffered from malnutrition—the perfect scenario for the spread of tuberculosis. Koch believed that what he found in the laboratory should make a difference in the world. In the first article that he wrote on tuberculosis, he stated his lifelong goal, "I have undertaken my investigations in the interests of public health and I hope the greatest benefits will accrue therefrom." Today, more than a century since Koch's discovery, despite the many marvels of medicine and public health, tuberculosis is still one of the most common preventable diseases of mankind, especially among the poor. Surely there is much more work to be done.

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Mycobacterium novocastrense–associated Pulmonary and Wound Infections

To the Editor: Although the clinical role of nontuberculous mycobacteria has long been appreciated (*1*), the high endemicity of tuberculosis (TB) in developing countries has overshadowed the emergence of these organisms. They are simply dismissed as being contaminants or are misidentified as *Mycobacterium tuberculosis* (*2*).

No report on *M. novocastrense* has been published since its original description in 1997 (*3*), except for a study in France by N'Guessan et al. (*4*). That study initiated speculation about the possible role of this bacterium in the etiology of human infection. We report isolation of *M. novocastrense* in 2 independent clinical cases—1 from tissue biopsy specimen of an apparently healthy adult, the other from bronchoalveolar lavage of an HIV-infected patient—that will cast light on the clinical relevance of this rare species.

Case-patient 1, a 60-year-old woman, was referred to a hospital because of high fever, productive cough, thoracic pain, and noticeable weight loss. After her husband's AIDS-related death in a state prison, HIV infection had been diagnosed in this case-patient. At admission, laboratory testing showed negative tuberculin skin test result, lymphopenia, an elevated C-reactive protein level of 76 mg/L, an erythrocyte sedimentation rate of 73 mm/h, a viral load of 500 copies/mL, and negative blood culture for bacterial growth. Her outpatient records indicated that she empirically was given numerous courses of antimicrobial drugs because of a provisional diagnosis of bacterial pneumonia, but her condition did not improve. Subsequent hospital referral was prompted by worsening of her symptoms. With a diagnosis of suspected TB, bronchoalveolar lavage fluid was collected. Direct microscopic examination of the specimen showed acid-fast bacilli with subsequent formation of typical colonies of a relatively rapidly growing photochromogenic *Mycobacterium* spp. on Löwenstein-Jensen (LJ) medium. The patient was treated with amikacin, and her condition markedly improved.

Case-patient 2, a 23-year-old woman, sought care for a 6-month history of soft tissue swelling in her left leg resulting from an accidental injury in a paddy field. The lesion had not been treated and subsequently increased in size. The large nodule self-ruptured and excreted yellow-pale pus. She was prescribed minocycline by her general practitioner, but the lesion did not resolve. When the patient arrived at the hospital, her clinical and laboratory assessments were normal. She had negative test results for HIV, hepatitis C, and hepatitis B infections. Erythrocyte sedimentation rate was 255 mm/h. Culture of a biopsy specimen from the cutaneous lesion on ordinary culture media was negative, although microscopic observation of the drainage fluid showed acid-fast bacilli. This finding was confirmed by a positive culture on LJ medium. A repeat specimen resulted in isolation of the same organism in pure culture. The patient was given amikacin and fully recovered in <1 month.

The isolates, i.e., HNTM1 and HNTM10, were subjected to preliminary identification and susceptibility to common antimycobacterial agents for rapidly growing mycobacteria according to standard procedures (*5*,*6*). The isolates were then subjected to molecular identification, which included PCR amplification of a genus-specific region of the 65-kDa heat shock protein (*hsp*) gene (*7*) and the direct sequence analysis of 16S rDNA, *hsp*65, and *rpo*B genes as described (*3*,*8*,*9*). GenBank accession numbers for the gene sequences of HNTM1 as a representative isolate determined in this study are HM807280–HM807282.

The isolates we observed by acid-fast staining of the specimens and recovered on LJ medium were a yellow pigmented photochromogenic species that grew rapidly at 25°C, 37°C, and 42°C. They grew on MacConkey agar without crystal violet and LJ medium containing 5% NaCl; were positive for semiquantitative catalase, arylsulfatase activity in 14 days, and nitrate reduction; and were negative for urease activity, niacin production, tellurite and Tween hydrolysis, heat-stable (68°C) catalase, and iron uptake. They were susceptible to amikacin, clarithromycin, doxycycline, sulfamethoxazole, streptomycin, imipenem, ciprofloxacin, isoniazid, and ethambutol but resistant to rifampin.

The PCR amplification of a 228-bp genus-specific fragment of the *hsp*65 gene reliably confirmed that the isolates belonged to the genus *Mycobacterium*. The almost complete 16S rDNA gene sequence (1,476 bp) and partial sequences of *hsp65* and *rpoB* genes of the isolates showed the highest similarity of 99.86%, 99.45%, and 99.7% with that of *M. novocastrense* reference strain. These values correspond to 2-nt differences for each gene.

Our report of 2 independent clinical cases might support evidence the clinical relevance of *M. novocastrense*. Our findings show that *M. novocastrense*, however rare its incidence might be, can cause infection in healthy and immunocompromised patients. However, because of the complexity of identifying nontuberculous mycobacteria, emphasis should be placed on the quality of regional laboratories for TB in developing countries to differentiate isolates to the species level.

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Geographic Expansion of Buruli Ulcer Disease, Cameroon

To the Editor: Buruli ulcer disease (BU) is a necrotizing skin disease caused by *Mycobacterium ulcerans* that affects mostly children in humid, tropical areas (*1*). The exact mode of *M. ulcerans* transmission remains unclear, although the role of water bugs has been supported by various observations and experimental studies (*2*,*3*). We report the identification of a new BU-endemic area in Cameroon, the Bankim district, and specify ecologic and clinical characteristics of *M. ulcerans* infection in this area. These characteristics hint at the possible role of environmental changes (building of a dam several years ago) in the expansion of BU in this area.

Since 1969, only 1 BU-endemic area in Cameroon has been described: the Nyong River basin, where equatorial forest predominates (*4*). In 2004, clinically suspected cases of BU in the district of Bankim have been reported (*5*). This region differs from the first BU-endemic area by geography and climate. Representing a transition between forested south and savanna north, this area has benefited from the building of a dam on the Mape River in 1989, which created an artificial lake of 3.2 billion m^3 capacity.

From January 2007 through June 2009, all cases of skin lesions evocative of active BU were recorded as BU probable cases according to World Health Organization guidelines (*6*). During this period, 195 clinically suspected cases were reported from the Bankim health district (Figure). The overall median age for these 195 patients was 19.5 years (interquartile range 10–37 years). No significant difference in age was found according to gender, but a significant trend of decreasing overall median age was found (20 years in 2007 to 12 years in 2009. The most frequent type of lesion was ulcer. Since March 2009, the Centre Pasteur of Cameroon has performed laboratory confirmation for suspected BU cases: microscopic examination for acid-fast bacilli, culture, and *M. ulcerans* DNA detection by PCR (*6*). From April through June 2009, of 34 consecutive samples tested in the reference laboratory, 10 were positive for *M. ulcerans* by at least microscopy and PCR.

Whether BU is emerging in Bankim or is just a newly recognized preexisting disease is difficult to establish. However, that the incidence of BU in the region is increasing is unquestionable. The decreasing median age of patients since 2007 might be consistent with emergence of BU as a new disease in Bankim. This observation could suggest either an increasing level of acquired immunity in the population, leading to protection correlated with age, or the expansion of risky sites for human infection with *M. ulcerans*.

During 1 week in January 2008, water bugs were collected from the artificial lake and water bodies located within or close to each community. A previously described sampling method was used (*2*). To detect *M.*

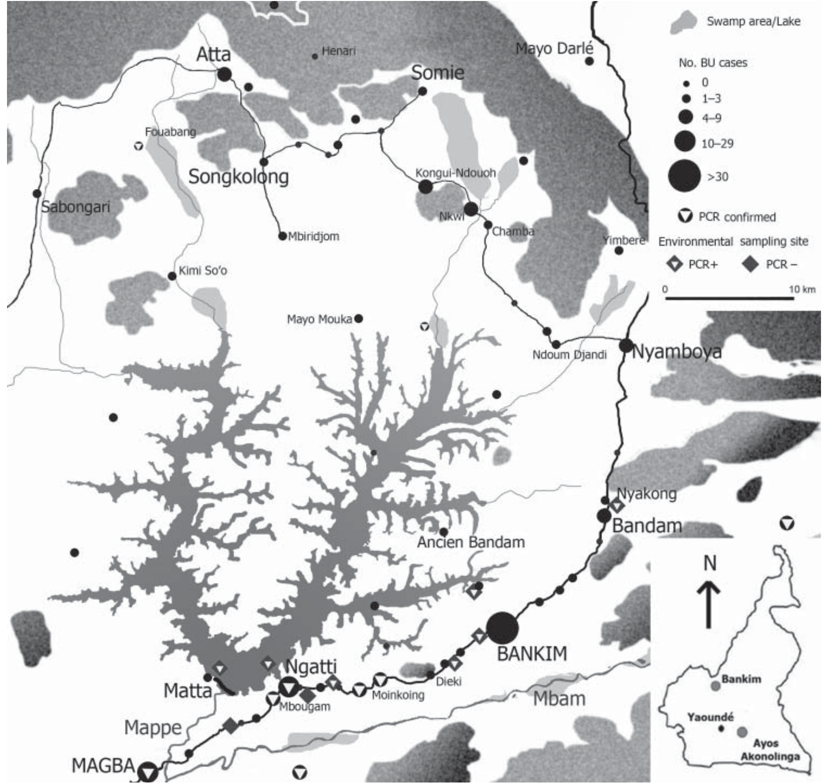

Figure. Distribution of Buruli ulcer (BU) patients reported January 2007–June 2009, and of water bodies with aquatic bugs harboring *Mycobacterium ulcerans,* Cameroon. Inset, Bankim area. A color version of this figure is available online (www.cdc.gov/EID/content/17/3/551-F.htm).

ulcerans DNA, we pooled the insects per family in groups of up to 10. Moreover, 99 members of the families Belostomatidae and Naucoridae were kept alive for saliva collection (*2*). The DNA of insect pool homogenized tissues and individual saliva samples were purified. We then searched for *M. ulcerans* molecular signatures (*2*). Among 1,349 insect specimens, 8 from the aquatic Heteroptera families were identified, and 12 (5%) of 244 insect pools were *M. ulcerans* positive. *M. ulcerans*–positive saliva was found in 11 (18%) of 61 insects in the family Belostomatidae and in 3 (8%) of 38 in the family Naucoridae. Water bodies where *M. ulcerans*–positive insects were collected are shown in the Figure.

The emergence of BU may be a consequence of the marked changes in the environment caused by the building of the dam. Elsewhere, human environmental modifications such as construction of dams have been linked with increased incidence of BU (*1*). The main visible environmental effect is the large amount of flooded farmland. According to the seasons, the reservoir margins change the milieu of swamps and meadows. All these modifications affect plant and animal resources in the reservoir area by favoring rapid growth of aquatic macrophyte populations during reservoir filling, thus providing breeding sites for insects and leading to the extinction of area-endemic species and creation of new niches (*7*). These changes might favor development of *M. ulcerans* in biofilms on aquatic plants, which are then ingested by herbivorous animals, which are further prey for water bug predators, hosts, and possible vectors of *M. ulcerans* (*8*,*9*). The water bugs that were most frequently trapped and colonized by *M. ulcerans* (families Belostomatidae, Naucoridae, Nepidae, Notonectidae) are carnivorous and able to bite humans (*10*).

Our study confirms expansion of BU in Cameroon. To facilitate detection of new BU foci, and to improve patient treatment (medical, surgical, rehabilitative), health care workers involved in tuberculosis/leprosy control programs should be educated about BU.

Acknowledgments

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Risk for *Mycobacterium celatum* Infection from Ferret

To the Editor: *Mycobacterium celatum* belongs to the group called "mycobacteria other than tuberculosis"; it is characterized by slow growth and a slender, rod-shaped form (0.25–0.5 × 0.5–13.0 μm). The cells are acid fast and do not form cords or branches. The species name, *celatum*, which means hidden or concealed, refers to the problem of phenotypically distinguishing the species from other mycobacteria, especially *M. xenopi. M. celatum* was first described in 1993 as a pathogen in persons with AIDS (*1*). Until now, few cases in humans have been reported; those cases were predominantly disseminated mycobacteriosis in immunocompromised patients (mainly those with AIDS), but they have also occurred in immunocompetent persons (*1,2*). For animals, 1 case of *M. celatum* infection in a ferret has been described (*3*). We describe another case in a ferret, with possible transmission to a human.

In 2009, a 3-year-old, neutered male, domestic ferret was examined in a veterinary clinic in Nuremberg, Germany, for a 5-month history of coughing, recent weight loss, reduced general condition, vomiting, and mild diarrhea. A chest radiograph showed multiple nodular densities in the lungs. Because of a poor prognosis, the ferret was euthanized. Necropsy was performed at the Institute of Veterinary Pathology in Munich. The lungs contained multifocal firm, light brown nodules, 6–10 mm in diameter (Figure, panel A). Spleen and lymph nodes (cervical, retropharyngeal, bronchial, gastric, mesenterial, popliteal) were enlarged. Histologic examination of lung, lymph nodes, spleen, liver, and brain showed granulomatous inflammation with predominantly macrophages, epithelioid cells (in the lung, including bronchioles), and some multinucleated giant cells. Several acid-fast bacilli were visible with Ziehl-Neelsen staining, mainly intracytoplasmically in epithelioid cells (including those of bronchioles) (Figure, panel B).

Conventional mycobacterial culture and PCR were used to look for mycobacteria in the lung, spleen, and lymph nodes. For culture, the material was homogenized, decontaminated, and spread onto solid Loewenstein-Jensen agar and injected into a liquid culture (Mycobacteria Growth Indicator Tube; Becton Dickinson, Heidelberg, Germany) for automated detection of mycobacterial growth.

DNA was extracted from the homogenized tissue by using the QiaAmp DNA Mini Kit (QIAGEN, Hilden, Germany), and a 510-bp fragment at the 5′ end of the ribosomal 16S rDNA was amplified as described (*4*). The amplified fragment of the expected length was sequenced, and data were analyzed by using the Integrated Database Network System (SmartGene Services, Lausanne, Switzerland; www.smartgene.com). The resulting sequence was clearly interpretable and unambiguously assigned to *M. celatum*; sequence identity to GenBank accession no. Z46664 was complete except for 1 mismatch in bp 490. Minor sequence diversity in the *M. celatum* 16S rDNA gene has been documented (*5*). The most closely related species, *M. kyorinense*, differs substantially, having 11 mismatches within the 16S rDNA gene (*6*). Species identity was

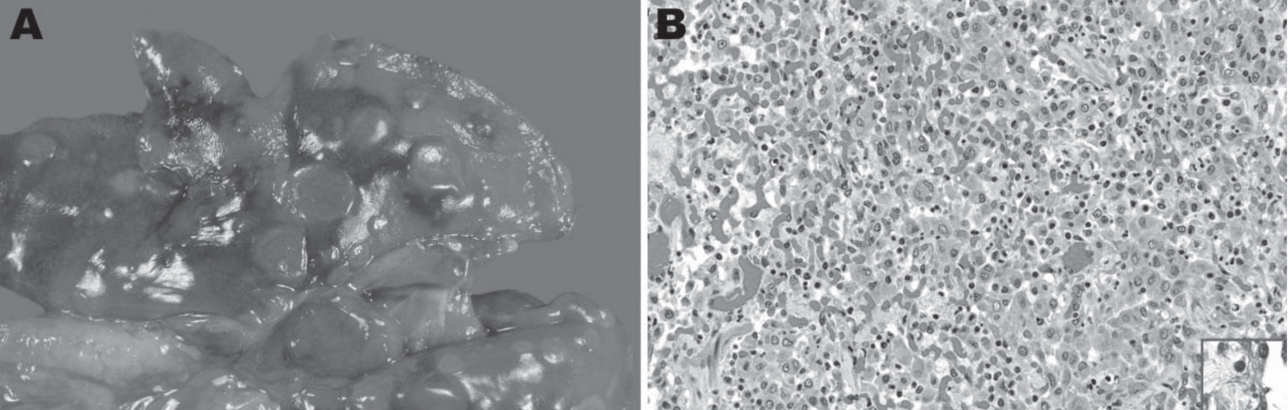

Figure. Appearance of tissue from 3-year-old, neutered male, domestic ferret with *Mycobacterium celatum* infection. A) Gross appearance: multiple, round foci over lungs. B) Histologic appearance, granulomatous pneumonia: alveoli filled with foamy macrophages, epithelioid cells, and a multinucleated giant cell; also mild interstitial infiltration with lymphocytes, plasma cells, and neutrophils. Hematoxylin and eosin staining, original magnification x200. Inset, slender, rod-shaped, acid-fast bacilli in the cytoplasm of epithelioid cells; Ziehl-Neelsen staining, original magnification x400. A color version of this figure is available online (www.cdc.gov/EID/content/17/3/553-F.htm).

further supported by phylogenetic analyses of the *hsp65* (*7*), *rpoB* (*8*), and *sodA* genes (*9*).

After 14 days of incubation, the solid and liquid cultures showed growth of acid-fast bacilli. Further identification at the Mycobacteria Reference Laboratory of the Bavarian Health and Food Safety Authority (Oberschleissheim, Germany) confirmed the molecular species typing results of *M. celatum*.

In Europe, naturally occurring mycobacterial infections in ferrets are rare; but in New Zealand, *M. bovis* or *M. avium complex* infections in ferrets are common (*10*). For ferrets, clinically relevant mycobacteria species are *M. genavense* and *M. microti*, among others. In humans, *M. celatum* mostly affects immunocompromised hosts with developing pneumonia or disseminated mycobacteriosis (*1*); inflammation in infected immunocompetent hosts is usually limited to the lungs or lymph nodes (*2*).

The ferret reported here had disseminated mycobacteriosis with no evidence of immunosuppression. In this respect, the clinical response of ferrets seems to differ from that in humans. The question of zoonotic risk remains. The ferret's owner had had a cough for a long time, but radiography and sputum analysis showed no evidence of infection. However, these findings do not rule out mycobacteriosis because the owner had received unknown antimicrobial drug therapy before the samples for microbiology were collected. In general, potential transmission of mycobacteria should not be underestimated; in this case, intracytoplasmic bacilli were detected in the bronchioli of the ferret's lung, making airborne spread of mycobacteria and infection of humans possible. The source of the primary infection in the ferret is not clear, but the dominant lesions in the lungs suggest it was airborne.

M. celatum infection must be strongly considered as a differential diagnosis in ferrets with pneumonia and generalized lymphatic hyperplasia. PCR and molecular species typing by 16S rDNA sequencing seem to be essential for an early and definitive diagnosis (*1*). The zoonotic risk for *M. celatum* infection in immunocompromised as well as immunocompetent persons should be kept in mind, considering the possible airborne transmission and the close contact between the animals and their owners.

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Human Infection with *Pseudoterranova azarasi* Roundworm

To the Editor: Eating raw or undercooked marine fish may lead to infection with several helminths. Members of the *Pseudoterranova decipiens* species complex are the second most common nematodes found in humans (most common are nematodes of the *Anisakis simplex* complex) (*1,2*). The *P. decipiens* species complex consists of at least 5 sibling species (genetically but not morphologically distinguishable): *P. decipiens* sensu stricto, *P. azarasi, P. cattani, P. krabbei*, and *P. bulbosa*. In northern Japan, human infection with *Pseudoterranova* spp. is not rare; by the mid-1990s, as many as 769 cases had been reported (*3*). Pseudoterranovosis has also been encountered in North and South America and Europe (*4,5*). However, possible biologic and geographic differences of the sibling species of genus *Pseudoterranova* in relation to human infection remain unknown. We report a case of pseudoterranovosis for which the sibling species was confirmed as *P. azarasi*.

In 2009, a woman in Japan coughed up a nematode and expelled it through her mouth. Her medical history was unremarkable, and she had not traveled abroad for the past few years. Measurements of the worm were as follows: body 35 mm long and 0.85 mm wide; esophagus 1.88 mm long; ventriculus 1.05 mm long; and intestinal cecum 0.95 mm long, extending anteriorly along the ventriculus. The anterior end of the worm contained 3 lips. The tail was conical, 0.21 mm long, and had a small, knob-like process at the posterior end. On the basis of morpohologic features, the worm was identified as a 4th-stage larva of *P. decipiens* (sensu lato) roundworms.

DNA was extracted from the isolate from the patient (clinical isolate) and from isolates of *P. decipiens* (sensu lato) larvae from Pacific cod purchased at a local market. PCR was performed and the amplification products were directly sequenced. Primers used were 5′-CCGGGCAAA AGTCGTAACAA-3′ and 5′-ATATG CTTAAATTCAGCGGGT-3′ for a region that spans the internal transcribed spacer (ITS) 1, ITS2, and 5.8S rRNA; 5′-CTACTACTAAGAATTTGCGT-3′ and 5′-AATCCAAATACTTACGAG GA-3′ for cytochrome oxidase subunit 1; and 5′-CAGCGTATTGGTCCT AATAA-3′ and 5′-AGCATAAACAAA AGTAAACTCA-3′ for NADH dehydrogenase subunit 1.

Nucleotide sequences for the clinical and Pacific cod isolates (GenBank accession nos. AB576756–AB576761) were compared with those in DNA databases available to the public. The ITS1 and ITS2 sequences of the clinical and Pacific cod isolates were identical to those of *P. azarasi* roundworms; however, the ITS sequences of these 2 isolates also showed close similarity to those of *P. decipiens* (sensu stricto) worms and differed by only 1 nt in ITS2. However, phylogenetic tree analyses of NADH dehydrogenase subunit 1 and cytochrome oxidase subunit 1 sequences showed that the clinical and Pacific cod roundworm isolates clustered with *P. azarasi* and were clearly distinguished from *P. decipiens* (sensu stricto), showing that the clinical isolate belonged to *P. azarasi* (Figure).

Geographic distribution of the 5 sibling species of the *P. decipiens* complex differs somewhat. *P. azarasi* and *P. bulbosa* are found in northwestern Pacific (including Japan), *P. decipiens* (sensu stricto) and *P. krabbei* in northeastern Atlantic, *P. decipiens* (sensu stricto) in northwestern Atlantic, and *P. cattani* in southeastern Pacific waters (*1*). Given this distribution, it is not surprising

that the clinical isolate and the larvae from the Pacific cod were identified as *P. azarasi*, 1 of 2 species found in water near Japan. Adult worms live in the intestines of seals and sea lions, and infective larvae live in the tissues of various marine fish, including cod, pollack, and smelt (*1*).

In Japan, most patients infected with *Pseudoterranova* spp. have acute or subacute abdominal pain, and larvae are extracted from the stomach endoscopically. However, for some patients, diagnosis is made when 4th-stage larvae are expelled from the mouth, indicating that the larvae developed from the 3rd to 4th stage during the time of infection, as did the worm reported here. Expulsion of *Pseudoterranova* spp. larvae from the mouth in the absence of severe gastric symptoms occurs more commonly in Chile (*5*). Whether the varied symptoms triggered by infection with *Pseudoterranova* spp. larvae reflect different responses of individual hosts to the worms or whether the pathogenicity of *Pseudoterranova* spp. in humans differs among worm species remains to be elucidated.

Because of the increasing worldwide popularity of eating sushi and sashimi made of raw marine fish, consumers should be made aware of the possible risk for fish-borne parasitoses. Freezing and storing fish at –20°C for 7 days or freezing at –35°C until solid and storing at –35°C for 15 hours is sufficient to kill parasites (*6*).

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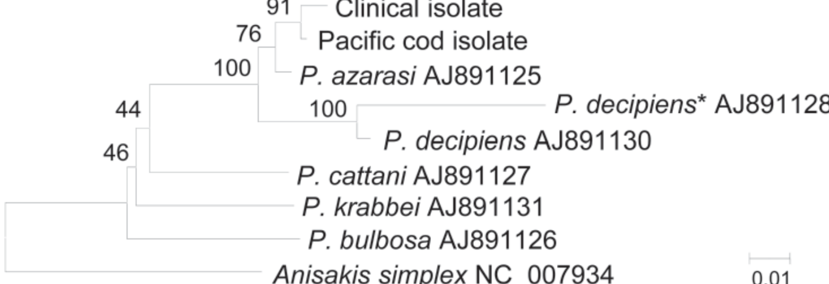

Figure. Phylogenetic analysis of members of *Pseudoterranova decipiens* species complex roundworms. Genetic relationships between NADH dehydrogenase subunit 1 sequences in clinical and Pacific cod isolates and species were inferred by using the neighbor-joining method. Bootstrap values (1,000 replicates) are shown next to the branches. The final dataset contained 498 positions. **P. decipiens* sensu stricto. Scale bar indicates nucleotide substitutions per site.

Mycobacterium mageritense Pulmonary Disease in Patient with Compromised Immune System

To the Editor: *Mycobacterium mageritense* is one of the rapidly growing mycobacteria (RGM). It was first isolated in Spain in 1987, described as a new species in 1997 by Domenech et al. (*1*), and first described and associated with disease in the United States in 2002 (*2*). In the 2002 report, 6 isolates were recovered from sputum, a bronchoscopy sample, a wound infection after liposuction, the blood of an immunosuppressed patient with a central catheter and sepsis, a patient with severe sinusitis, and from a wound infection in a patient who had probable osteomyelitis after fixation of an open fracture. It has since

been reported as a cause of water-related skin and soft tissue infections (*3*,*4*). A study from Japan in 2007 reported recovery of *M. mageritense* from the sputum of a woman with noncaseating granulomas by transbronchial biopsy who improved without therapy (*5*). We describe a case of *M. mageritense* pneumonia in an immunocompromised patient.

In 2009, a 54-year-old woman was admitted to the hospital in Austin, Texas, with a 5-day history of upper back pain and occasional hemoptysis and yellow sputum production. She had a long history of systemic lupus erythematosus and associated nephritis and vasculitis, rheumatoid arthritis, hypothyroidism, sleep apnea, and hepatitis C infection. She was taking prednisone 15 mg/day at the time of admission.

Five months earlier, organizing pneumonia was diagnosed in the patient by computed tomography–guided lung biopsy of a pleura-based mass; special stains and cultures on tissue for acid-fast bacilli (AFB), other bacteria, and fungi were negative. She was readmitted several times over subsequent months and treated with various antimicrobial agents and corticosteroids but did not show clinical or radiographic improvement. Chest computed tomographic scan performed at admission again demonstrated bilateral lung masses and infiltrates, with new areas of necrosis. A second needle biopsy sample showed chronic inflammation with a histiocytic reaction and negative stains for AFB and fungi, but it was deemed nondiagnostic. Subsequent open lung biopsy sample showed necrotizing granulomatous inflammation with possible vascular involvement suggestive of Wegener granulomatosis.

Fite staining showed rare clusters of AFB within the granulomas. The postoperative course was complicated by a multiloculated left pleural effusion. AFB smear of pleural fluid obtained from video-assisted thoracoscopy showed 1–5 bacilli per high power field. Cultures of lung tissue and pleural fluid grew mycobacteria initially identified as *M. fortuitum* group but subsequently identified as *M. mageritense* by PCR followed by restriction enzyme analysis of the 65-kDa heat-shock protein (*hsp65*) (*6*). Results of susceptibility testing by broth microdilution are shown in the Table.

Testing for Wegener granulomatosis by antineutrophilic cytoplasmic and myeloperoxidase antibody yielded negative results. Imipenem and amikacin were prescribed, and gradual resolution of clinical signs and symptoms was observed. Oral linezolid and trimethoprim/sulfamethoxazole were prescribed at discharge. Chest radiographs taken 4 months after the open lung biopsy showed resolution of the masses.

The isolate was a nonpigmented RGM that matched the American Type Culture Collection (Manassas, VA, USA) type strain and 10 published clinical isolates of *M. mageritense* by PCR restriction enzyme analysis of the 65-kDa *hsp* gene (*6*). By gene sequencing of region V of the RNA polymerase (*rpoB*) gene, it exhibited 99.7% identity to the GenBank type strain sequence of *M. mageritense* (acceptable interspecies relatedness for this sequence is ≥98.5% identity) (*8*). The most closely related species determined by using this sequence and previously submitted sequences were other *M. fortuitum* species: *M. porcinum* (94% sequence identity), *M. wolinskyi* (94%), and *M. peregrinum* (93%).

Susceptibility testing of 23 clinical isolates of *M. mageritense* from the United States previously submitted to the Mycobacteria/Nocardia Research Laboratory (University of Texas Health Science Center, Tyler, TX, USA) and identified by *hsp65* PCR restriction analysis (*6*,*7*) was performed (Table). These results confirmed the potential utility of the drugs used in this case for future cases.

M. mageritense has not been reported as a cause of pulmonary

Table. In vitro activity of 23 isolates of *Mycobacterium mageritense*, United States, 2009*

Antimicrobial agent	No. isolates tested	MICs of current isolate, μg/mL	Intermediate breakpoint, μg/mL	MIC range, μg/mL	MIC_{50}, μg/mL	MIC_{90}, μg/mL	% S/I
Amikacin	23	8	32	≤1–32	16	32	100
Cefoxitin	23	16	32–64	≤8–256	32	64	91
Ciprofloxacin	23	0.25	2	≤0.25–0.5	0.25	0.5	100
Clarithromycin†	23	8	4	1–>64	>32	>64	4
Doxycycline	22	1	2–8	0.25–>64	8	>32	50
Imipenem	22	4	8	≤0.5–8	2	4	100
Linezolid	22	4	16	≤2–16	4	8	100
Sulfamethoxazole	21	4	32	≤2–32	8	32	100
Trimethoprim/sulfamethoxazole	6	1/19	2/38‡	≤0.25/4.8– 2/38	0.5/9.5	2/38	100
Tobramycin	23	≤2	8	2–64	>16	>32	30
Tigecycline	5	0.12	–§	≤0.03–0.12	0.06	0.12	NA

*Includes 6 isolates previously reported (*2*). S, susceptible; I, intermediate; NA, not available.
†Three days' incubation.
‡Proposed breakpoint (*7*).
§No Clinical and Laboratory Standards Institute breakpoints established for tigecycline.

disease in an immunocompromised patient. However, most cases of *M. fortuitum* pneumonia were reported before the use of molecular technology for species identification. Newer species such as *M. mageritense* resemble *M. fortuitum* and would not have been differentiated without this method.

Our patient met the criteria for diagnosing nontuberculous mycobacterial lung disease as established by the American Thoracic Society and the Infectious Diseases Society of America (*9*). Her therapeutic response also supports a cause-and-effect relationship.

The identity of an RGM isolate as *M. mageritense* may be suspected by its unusual antimicrobial drug susceptibility pattern, which showed an intermediate MIC to amikacin and resistance to clarithromycin at 3 days (Table). However, definitive identification requires molecular methods. Previous studies have shown that *M. mageritense* contains an inducible erythromycin methylase gene (*erm* 40) that confers macrolide resistance (*10*). The use of molecular studies and greater attention to susceptibility patterns should enable increased recognition of *M. mageritense* as a human pathogen.

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Extensively Drug-Resistant Tuberculosis, China

To the Editor: The prevalence of drug-resistant tuberculosis (TB) is a serious problem in the People's Republic of China. China is 1 of 22 countries with the highest incidence of TB (*1*). It is also 1 of 27 countries with the highest incidence of multidrug-resistant TB (MDR TB) and extensively drug-resistant TB (XDR TB). According to the national baseline survey on TB in 2007 and 2008, the frequency of MDR TB among pulmonary TB patients in China was 8.3%. We estimate that there are 120,000 new cases of MDR TB in China per year, which accounts for 24.0% of new cases worldwide (510,000) per year.

XDR TB has recently emerged as a global public health problem (*2*). It is defined as TB with resistance to at least isoniazid, rifampin, a fluoroquinolone, and 1 of 3 injectable second-line drugs (amikacin, kanamycin, or capreomycin). XDR TB is a type of MDR TB that shows resistance to isoniazid and rifampin. Recent reports on current prevalence of XDR TB (*3*,*4*) indicate that China now has the second highest incidence of MDR TB worldwide. However, there is no information available on XDR TB in China.

To obtain information on XDR TB in China, we conducted a study

at Shanghai Pulmonary Hospital. It is the only specialized hospital for TB in Shanghai and plays a major role in treating TB patients and providing state-of- the-art treatment. Most patients referred to this hospital have been previously treated or have recurrent TB. Therefore, higher rates of MDR TB and XDR TB are expected in this setting, which is not comparable to community or multicenter-based studies.

Patients with culture-proven MDR TB during January 2008–June 2009 were retrospectively evaluated. All patients were HIV negative. Drug susceptibility testing was conducted for culture-positive isolates by using the BACTEC 960 System (Becton Dickinson, Franklin Lakes, NJ, USA) at concentrations of 0.1 μg/mL for isoniazid, 1 μg/mL for rifampin, 5 μg/mL for ethambutol, 1 μg/mL for streptomycin, 2.5 μg/mL for capreomycin, 1μg/mL for amikacin, and 2 μg/mL for ofloxacin.

Among 518 strains that were culture positive for *Mycobacterium tuberculosis*, 350 (67.6%) were drug resistant and 168 (32.4%) were drug sensitive. A total of 217 (41.9%) of 518 strains were classified as MDR and accounted for 62.0% of drug-resistant strains. Among 217 MDR strains, 45 (20.7%) were from patients who had a new diagnosis of TB, and 172 (79.3%) were from patients whose medical history included treatment for TB for $\geq$4 weeks. A total of 65 (12.6%) strains were XDR, of which 51 were from patients previously treated. These strains accounted for 18.6% of drug-resistant strains and 30.0% of MDR strains.

Of 217 MDR isolates, 217 (100.0%), 217 (100.0%), 172 (79.3%), 175 (80.6%), 170 (78.3%), 68 (31.3%), and 69 (31.8%) were resistant to isoniazid, rifampicin, streptomycin, ethambutol, ofloxacin, capreomycin, and amikacin, respectively. Of 65 XDR isolates, 65 (100.0%), 65 (100.0%), 61 (93.9%), 60 (92.3%), 65 (100.0%), 60 (92.3%), and 60 (92.3%) were resistant to isoniazid, rifampicin, streptomycin, ethambutol, ofloxacin, capreomycin, and amikacin, respectively.

Our results indicate that 30.0% of MDR strains were XDR strains. Although our study was conducted in only 1 hospital, this prevalence of XDR strains indicates that XDR TB in China is a serious concern. A total of 78.3% of MDR isolates were resistant to ofloxacin, which is higher than rates reported for South Korea (42.8%) (*5*) and Taiwan (16.6%) (*6*). Population-based studies have reported lower frequencies of XDR strains among MDR strains; 9.9% for 14 qualified reference laboratories (*7*), 5.3% for South Korea (*8*), and 23.9% for South Africa among patients co-infected with HIV and TB (*9*).

In our study, 2 factors may have contributed to high drug-resistance rates. First, fluoroquinolones have been widely used for treatment of respiratory tract bacterial infections because of their efficacy and mild adverse reactions. Second, we also prescribed fluoroquinolones for treatment of patients with drug-resistant TB and some patients with drug-sensitive TB who could not tolerate first-line anti-TB drugs. More than 90% of patients with XDR TB had strains resistant to streptomycin, ethambutol, capreomycin, and amikacin, which was higher than rates reported in other studies (*5,9,10*). Currently, anti-TB medications in China for treatment of patients with XDR TB are scarce. This scarcity has resulted in poor treatment outcomes in patients with XDR TB.

One limitation of our study is that we investigated patients at only 1 specialized TB hospital in Shanghai. Therefore, data are not representative for the general population. A community-based multicenter study is needed to determine the true prevalence of XDR TB in China. Nevertheless, our study confirms that the prevalence of MDR TB and XDR TB is high in some areas. It also emphasizes the need to increase TB prevention and therapy, educate society about TB, implement modern TB control strategies, and strengthen basic and clinical research to curb the spread of MDR TB and XDR TB.

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Clade 2.3.2 Avian Influenza Virus (H5N1), Qinghai Lake Region, China, 2009–2010

To the Editor: In 2005, a large population of wild migratory birds was infected with highly pathogenic avian influenza (HPAI) virus (H5N1) in the Qinghai Lake region of western People's Republic of China, resulting in the death of ≈10,000 birds (*1*,*2*). On the basis of phylogenetic analysis of the hemagglutinin (HA) gene, the virus was classified as clade 2.2 according to the World Health Organization guidelines. Subsequently, viruses from this clade were found in Mongolia, Russia, Europe, and Africa along the migratory flyways of birds (*3*,*4*). This unique distribution of the same clade of HPAI virus (H5N1) through different migratory routes indicates that migratory birds might play a global role in virus dissemination (*3*,*4*).

In 2006, viruses from the same clade were isolated in the Qinghai Lake region (*3*). Analysis of viral outbreaks along migratory flyways demonstrated a similar outbreak pattern for the past 4 years (2006–2009) (*5*). During that period, clade 2.2 avian influenza virus (H5N1) was isolated in China, Mongolia, Russia, Germany, Egypt, and Nigeria; all viruses were closely related to the Qinghai Lake virus. Despite the broad distribution of clade 2.2 viruses in migratory flyways, few isolates of clade 2.2 viruses in local domestic poultry were reported, especially in China (*6*). Outbreaks of these viruses were reported in poultry in Africa (*7*). The reason these viruses rarely cause outbreaks in poultry is unknown.

During May–June 2009 and 2010, several dead migratory birds were found in the Qinghai Lake region. Nine HPAI viruses (H5N1) were isolated in 2009 and 2 were isolated in 2010 from great cormorants (*Phalacrocorax carbo*), brown-headed gulls (*Chroicocephalus brunnicephalus*), great black-headed gulls (*Ichthyaetus ichthyaetus*), great-crested grebes (*Podiceps cristatus*), and bar-headed geese (*Anser indicus*) and serotyped as described (*3*). HA genes from all 11 isolates were subsequently amplified by using reverse transcription–PCR and sequenced.

Phylogenetic analysis of HA sequences and an additional HA gene sequence from the 2009 Qinghai Lake subtype H5N1 virus isolate from a great crested grebe (from the National Avian Influenza Virus Reference Laboratory, Harbin, China) (GenBank accession no. CY063318) showed that HA genes from all 12 viruses clustered as clade 2.3.2 (Figure); none clustered with clade 2.2 viruses. Additionally, the HA cleavage site in the new isolates is PQRERRRKRG, which is identical to that of clade 2.3.2 viruses. In clade 2.2, the cleavage site is PQRERRRKKRG.

A bootstrap (1,000×) maximum likelihood tree (*8*) also demonstrated that Qinghai 2009 and 2010 virus isolates are closely related to those isolated in Mongolia and Uvs Nuur Lake in 2009, as reported by Sharshov et al. (*5*). Qinghai Lake and Uvs Nuur Lake, which are found along the migratory flyway in central Asia, are major lakes for bird migration and breeding. Many birds fly from Qinghai Lake to Uvs Nuur Lake in the spring.

If one considers isolation date and bird species infected, viruses isolated in Mongolia and Russia and our isolates were likely transmitted between the 2 lake regions by bird migration. Moreover, HA sequences are closely related to viruses isolated from wild birds in Hong Kong and Japan during 2007–2008, which are the most recent isolates of clade 2.3.2 viruses before isolation of 2009 Qinghai Lake viruses. These results indicate that viruses in the Qinghai Lake region may be transmitted by wild birds along the migratory flyway in eastern

Asia. However, there is no evidence that avian influenza virus (H5N1) is transmitted from eastern Asian (inner China or across the Himalayas) to the Qinghai Lake region.

The 2009 and 2010 Qinghai Lake viruses are related to various viruses isolated from plateau pikas near Qinghai Lake (*9*). In 2007, clade 2.2 and clade 2.3.2 viruses were isolated from plateau pikas, but no clade 2.3.2 viruses were found in aquatic birds. Wild birds, pikas, and other animals near Qinghai Lake share the same environment, and viruses may be transmitted across species. However, surveillance data are limited for wild animals near Qinghai Lake. Therefore, further investigations need to be conducted to clarify relationships among birds, animals, and influenza viruses near Qinghai Lake.

Our results and those of Sharshov et al. (*5*) show that in 2009 HPAI virus (H5N1) began infecting birds along the migratory route near Qinghai Lake and changed from clade 2.2 viruses to clade 2.3 viruses. New outbreaks of HPAI viruses (H5N1) along this migratory flyway should be investigated.

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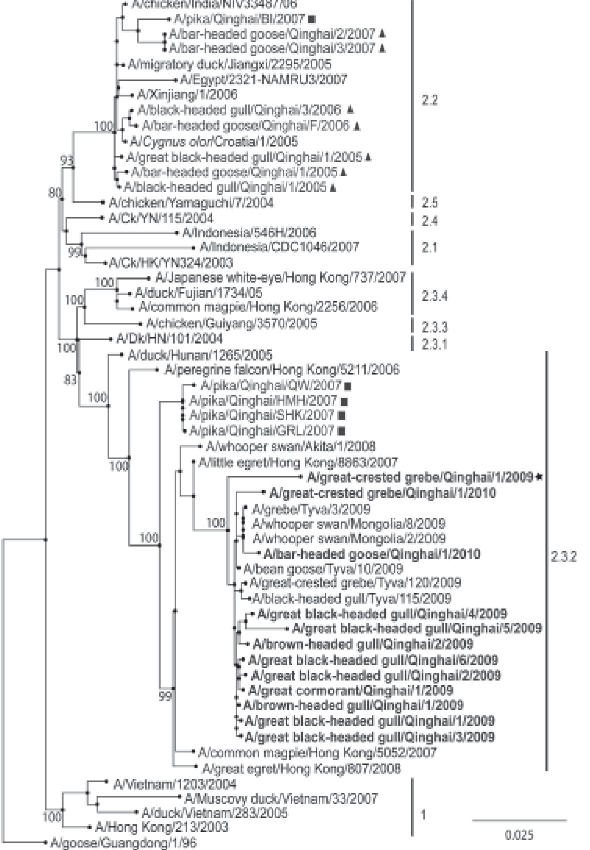

Figure. Bootstrapped (1,000×) maximum likelihood phylogenetic tree of hemagglutinin genes of avian influenza viruses (H5N1), People's Republic of China, 2009–2010. Viruses isolated from the plateau pika near Qinghai Lake are indicated by squares; viruses isolated from wild birds in Qinghai Lake Region during 2005–2007 are indicated by triangles; 2009 Qinghai virus submitted to GenBank by the National Avian Influenza Virus Reference Laboratory (Harbin, China) is indicated by the star and in **boldface**; and viruses isolated in 2009 and 2010 in the Qinghai Lake Region are indicated in **boldface**, Clade numbers are indicated on the right. NAMRU3, Naval Medical Research Unit 3; Ck, chicken; Dk, duck. Scale bar indicates nucleotide substitutions per site.

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Diagnosis and Treatment of Tuberculosis in the Private Sector, Vietnam

To the Editor: In many countries, the private sector (practitioners not employed by government and nongovernment institutions, e.g., hospitals, pharmacies) is a major source of care, even for poor persons, and the area where services for the public are widely available (*1,2*). However, little information is available from high-incidence countries about the role of the private sector in tuberculosis (TB) detection and treatment (*3*). In Vietnam, ≤40% of all TB cases in Ho Chi Minh City (the largest city in Vietnam and with the highest rate of economic growth in the country) were estimated to be treated in the private sector (*4*), and half of all patients with a diagnosis of TB in the public sector (National Tuberculosis Program [NTP]) in Ho Chi Minh City initially sought help in the private sector (*5*). However, this estimate does not reflect private care in the entire country.

In 2006–2007, a countrywide TB prevalence survey was conducted in Vietnam (*6*) in which data were obtained for previous TB treatment. This survey provided an opportunity to calculate a nationally representative estimate of the proportion of TB cases treated in the private sector and to investigate demographic characteristics of persons choosing treatment in this sector.

The study was reviewed and approved by the Research Board of the Vietnam National Lung Hospital. Details of survey methods have been reported (*6*). All eligible persons were screened to identify suspected cases of TB by using a short, structured, screening questionnaire and chest radiograph. Persons with suspected TB were those who reported persistent productive cough, who had radiographic abnormalities suggestive of TB, or who received TB treatment either currently or in the 2 years preceding the survey. Persons had an in-depth interview that included questions on where they were treated for TB. Assessment of socioeconomic status was based on 9 household characteristics (*7*).

Missing data were imputed by using multiple imputation methods, assuming that these data were missing at random to adjust for nonparticipation and missing data on facility of TB treatment (*8*). We used the ice and mi commands in Stata version 11 software (StataCorp LP, College Station, TX, USA), which included age, area, zone, and socioeconomic status.

Of the 103,924 eligible persons in selected districts, 94,179 (91%) were screened, 7,498 were identified as having suspected TB, and 407 reported having been recently treated for TB: 316 (77.6%) in public health facilities (PHFs) reporting cases to the NTP, 8 (2.0%) in PHFs not reporting cases to the NTP, and 29 (7.1%) in private health care facilities not reporting to the NTP. Fifty-four (13.3%) did not provide information about where they were treated. Multiple imputation led to adjusted proportions of 88.9%, 2.9%, and 8.2%, respectively. Sensitivity analyses, which assigned 54 persons with missing data for location of TB treatment to PHFs or private clinics, resulted in a range of 7.1%–20.3% for private sector treatment.

Characteristics of participants by type of facility where they received TB treatment are shown in the Table. Women, younger persons, and residents of southern Vietnam were more likely to seek treatment in the private sector. Urban populations and those with the highest socioeconomic status were most likely to seek private care, but these differences were not significant (Table).

Table. Characteristics of patients treated for tuberculosis at time of prevalence survey (2006–2007) or in 2 preceding years, Vietnam*

Characteristic	Public health facilities reporting to NTP, no. (%)	Public health facilities not reporting to NTP, no. (%)	Private sector, no. (%)	OR (95% CI)
Total	316 (89.5)	8 (2.3)	29 (8.2)	NA†
Sex				
M	230 (92.4)	4 (1.6)	15 (6.0)	1
F	86 (82.7)	4 (3.8)	14 (13.5)	2.4‡ (1.0–5.6)
Age, y				
15–35	51 (79.7)	2 (3.1)	11 (17.2)	3.1 (1.3–7.4)
35–55	134 (93.1)	4 (2.8)	6 (4.2)	0.4 (0.1–0.9)
>55	131 (90.3)	2 (1.4)	12 (8.3)	1.0 (0.4–2.3)
Area				
Urban	95 (85.6)	2 (1.8)	14 (12.6)	2.2 (0.9–5.1)
Remote	59 (89.4)	2 (3.0)	5 (7.6)	0.9 (0.3–2.5)
Rural	162 (92.0)	4 (2.3)	10 (5.7)	0.5 (0.2–1.2)
Zone				
Northern	117 (93.6)	5 (4.0)	3 (2.4)	0.2 (0.0–0.7)
Central	50 (87.7)	1 (1.8)	6 (10.5)	1.4 (0.4–3.8)
Southern	149 (87.1)	2 (1.2)	20 (11.7)	2.5 (1.1–6.5)
Socioeconomic status§				
Lowest	104 (91.2)	2 (1.8)	8 (7.0)	0.8 (0.3–1.9)
Medium	85 (90.4)	2 (2.1)	7 (7.4)	0.9 (0.3–2.2)
Highest	103 (88.0)	2 (1.7)	12 (10.3)	1.5 (0.6–3.5)
No information	24 (85.7)	2 (7.1)	2 (7.1)	NA¶

*NTP, National Tuberculosis Program; OR, odds ratio, CI, confidence interval; NA, not available.
†Not available because ≥4 categories are needed for calculation of OR.
‡For difference between private and public sectors. All other comparisons were for differences between 1 group and the other groups combined.
§Based on a set of indicators (*7*) and expressed by tertiles of expenditure distribution among all survey participants.
¶Not available because there were only 28 persons with no information.

We estimated that 8.2% of persons with TB in Vietnam were treated in private clinics. Although sensitivity analysis showed a wide range around this estimate (7.1%–20.3%), our data suggest that private health care facilities treat a large proportion of TB patients. Use of the private sector was relatively high in southern Vietnam (11.7%), especially in southern urban areas (13.3%).

Taking into account observed patterns in our study (i.e., preference for private sector treatment among young persons, higher income groups, and those in urban areas) and general development in Vietnam, we expect that the private sector will provide increased diagnosis and treatment of TB. With availability of TB drugs in private pharmacies, (*9*) improved private TB care is needed (*10*) by establishing better collaboration and coordination between the NTP and the private sector through the public–private approach (*4*) and by improving and expanding reporting systems so that all facilities where TB patients are diagnosed and treated are included.

A strength of our study is that it included a nationally representative sample of previously treated TB patients because all who reported having been treated recently for TB were defined as persons with suspected TB. A limitation of our study is that it depended on self-reported TB treatment, which is prone to recall bias and has potentially led to underestimation because some participants may not have been accurate regarding previous or current episodes of TB and private sector treatment because of social desirability bias. The NTP in Vietnam needs to implement and improve public–private mix projects, and private practitioners need to be appropriately trained to report TB patients according to NTP guidelines.

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Fluoroquinolone-Resistant *Mycobacterium tuberculosis*, Pakistan, 2005–2009

To the Editor: Pakistan is 1 of 22 countries listed by the World Health Organization (WHO) as having a high incidence of tuberculosis (TB). We recently reported an increase in rates of multidrug-resistant (MDR) TB with emergence of extensively drug-resistant TB (*1*). Fluoroquinolone resistance is associated with worse outcome in patients with MDR TB (*2*). Recent evidence suggests emergence and increasing incidence of fluoroquinolone-resistant *Mycobacterium tuberculosis* from several countries, particularly in MDR strains (*3*). We present data from a tertiary care referral center laboratory in Pakistan to assess fluoroquinolone resistance in MDR TB strains during 2005–2009.

The Aga Khan University Hospital and its clinical laboratory have been accredited by the Joint Commission of International Accreditation and designated as a technical partner of the National TB Program. *M. tuberculosis* susceptibility testing is also periodically validated by the WHO Supranational Reference Laboratory network. The microbiology laboratory serves different cities across Pakistan with ≈180 peripheral collection units. Specimens for TB cultures are requested by physicians and received through passive collection and thus are not restricted to programmed surveys. All specimens received at each of the collection units are sent to the central laboratory in Karachi for culture and drug susceptibility testing (DST). Specimens reach the main laboratory within 24 hours after receipt.

During the past 4 years, the laboratory has received 12,000–15,000 specimens annually for *M. tuberculosis* culture; positivity rate has been 15%–20%. Culture and DST are performed at the laboratory in accordance with Clinical Laboratory Standards Institute and WHO recommendations, as described (*4*). During 2005–2008, fluoroquinolone susceptibilities for all MDR and polydrug-resistant isolates were determined by using ciprofloxacin (2 μg/mL). From 2009 onwards, fluoroquinolone susceptibilities were determined by using ofloxacin (2 μg/mL), and second-line DST was performed for all *M. tuberculosis* isolates.

During 2005–2009, a total of 11,263 cultures were reported positive for *M. tuberculosis*. Of these, 34.4% were MDR, and 50.1% were sensitive to all 4 first-line agents (isoniazid, rifampin, pyrazinamide, ethambutol). Because of inconsistencies in testing criteria for fluoroquinolones (fluoroquinolone testing being conducted primarily for MDR cases during 2005–2008), the overall fluoroquinolone-resistance rate could not be determined. However, for MDR strains, fluoroquinolone susceptibilities were consistently determined, and resistance rates increased from 17.41% in 2005 to 42.92% in 2009 ($p<0.001$, by χ^2 test for trend analysis) (Table).

A progressive increase in fluoroquinolone use and its association with increase in resistance against organisms other than *M. tuberculosis* have been reported from Pakistan (*5*). We report a progressive increase in fluoroquinolone resistance rate in MDR *M. tuberculosis* isolates

Table. Resistance patterns and fluoroquinolone resistance rates of *Mycobacterium tuberculosis* isolates from the Aga Khan University Hospital laboratory, Karachi, Pakistan, 2005–2009*

Year and isolates	Sensitive†	Multidrug resistant	Extensively drug resistant	Other resistance patterns‡	Total
2005					
Total	773	643	5	361	1,782
Fluoroquinolone resistant	NT	112 (17.41)	5 (100)	6 (1.66)	NA
2006					
Total	949	728	11	195	1,883
Fluoroquinolone resistant	NT	128 (17.58)	11 (100)	7 (3.58)	NA
2007					
Total	1,054	782	17	158	2,011
Fluoroquinolone resistant	NT	163 (20.84)	17 (100)	8 (5.06)	NA
2008					
Total	1,305	991	32	256	2,584
Fluoroquinolone resistant	NT	351 (35.41)	32 (100)	17 (6.64)	NA
2009					
Total	1,560	1,181	53	209	3,003
Fluoroquinolone resistant	48 (3.07)	507 (42.92)	53 (100)	23 (11)	631 (21.01)
p value§	NA	<0.001	NA	<0.001	NA
Total	5,641	4,325	118	1,179	11,263

*Values are no. (%) isolates in that category, except p values. NT, not tested; NA, not applicable.
†To isoniazid, rifampin, pyrazinamide, and ethambutol.
‡Other resistance patterns include isoniazid-monoresistant and polydrug-resistant isolates.
§χ^2 trend analysis.

during a 5-year period. This finding is consistent with those of several studies reporting fluoroquinolone resistance from the region. Agrawal et al. have recently reported an exponential increase in fluoroquinolone resistance in India from 3% in 1996 to 35% in 2004 (*6*). A significant increase in fluoroquinolone resistance from 7.7% to 20% in MDR TB was also reported from Taiwan (*7*); the authors correlated this finding with the inappropriate use of fluoroquinolones for managing TB rather than with fluoroquinolone misuse in the community. Another study from the United States and Canada reported 4.1% fluoroquinolone resistance in MDR TB strains (*8*).

In addition to detecting increasing fluoroquinolone resistance in MDR isolates, we have also detected fluoroquinolone resistance in non-MDR, polydrug-resistant *M. tuberculosis* isolates. Moreover, in 2009, a total of 3.1% of isolates susceptible to all first-line agents were fluoroquinolone resistant.

Although our dataset includes samples from throughout Pakistan, sampling limitations prevent us from deriving definite conclusions and generalizing results to the entire population of the country. A referral bias attributable to passive sampling exists because cases referred to our laboratory tend to be more complicated. Moreover, treatment history was not available for patients in our dataset; therefore, increased fluoroquinolone resistance could not be correlated with prior fluoroquinolone use. However, our findings have implications for therapy with fluoroquinolones for TB and other infections.

Fluoroquinolones are freely available as over-the-counter medications to the general population (*9*), which creates potential for misuse of fluoroquinolones by the general population for TB and several other infections, such as enteric fever and genitourinary infections. Furthermore, because national guidelines for treating enteric fever and genitourinary infections do not exist, these drugs are also overprescribed by physicians. We propose control of over-the-counter availability of fluoroquinolones and judicious use of this class of drugs by physicians to prevent further escalation in resistance rates in Pakistan.

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Hepatitis A Associated with Semidried Tomatoes, France, 2010

To the Editor: In January 2010, two clusters of nontraveler-associated hepatitis A were reported in 3 districts of southwestern France. A single IB strain of hepatitis A virus (HAV) was isolated (FR-2010-LOUR, GenBank accession no. GU646039). We conducted an investigation to describe the outbreak, identify the vehicle of transmission and source of infection, and propose appropriate control measures.

Cases were identified through mandatory notification or through the National Reference Centre for HAV. A total of 59 cases were identified: 49 confirmed cases (resident of France and infected with the outbreak strain) and 10 probable cases (resident of southwestern France and with a locally acquired infection positive for HAV immunoglobulin M against HAV with onset during November 1, 2009–February 28, 2010). Twelve (20%) persons were secondary case-patients (symptom onset 2–6 weeks after contact with a case-patient).

Twenty-eight (47.5%) case-patients were hospitalized, and all recovered. Case-patients were 7–54 years of age (median 31.5 years). The male:female ratio was 1.2:1. Cases were scattered throughout France, with 1 cluster each in Lot and Hautes-Pyrénées districts. Case-patients reported symptom onset during November 20, 2009–February 17, 2010 (Figure), with peaks during December 20, 2009–January 2, 2010, and January 24–30, 2010. The epidemiologic curve suggested a persistent common source of contamination, followed by person-to-person transmission. Of the 47 persons with nonsecondary cases (primary cases and cases that were not able to be classified), 27 (57%) reported having eaten in a sandwich shop. Twenty-four (51%) reported eating semidried tomatoes, 20 of whom reported purchasing semidried tomatoes in 1 of 3 different sandwich shop chains.

We conducted a case–control study of 30 nonsecondary case-patients with symptom onset during November 22, 2010–January 9, 2010; 109 controls (15–60 years of age living in the same district as case-patients and without histories of HAV infection or hepatitis A vaccination) were selected by random digit dialing. Exposures occurring 2–6 weeks before illness onset (case-patients) and before interview (controls) were recorded by telephone by using a standardized questionnaire. Logistic regression was performed (Stata 9.2; StataCorp LP, College Station, TX, USA); $p<0.05$ was considered statistically significant. Case-patients were more likely than controls to have eaten sandwiches or salads from a sandwich shop (age-adjusted odds ratio 29.1, 95% confidence interval 9.7–87.0) and to have eaten semidried tomatoes (age-adjusted odds ratio 8.5, 95% confidence interval 4.4–30.2).

HAV genotyping was performed as described (*1*). The epidemic strain FR-2010-LOUR was genotype IB. No identical strain was found in the National Reference Centre for HAV sequence database, even though IB strains represented one third of routinely isolated strains. The strain clustered significantly with sequences from patients returning from Turkey.

Trace-back investigations identified a supplier in France that imported frozen semidried tomatoes from Turkey and supplied the 3 sandwich shop chains. In France, the frozen semidried tomatoes were defrosted and processed with oil and herbs before distribution. No heat treatment, disinfection, or washing was conducted after defrosting. The period of distribution of 1 batch matched the estimated period of contamination of nonsecondary cases. This batch was no longer available at the supplier or at the sandwich shops for virologic analysis or for recall.

Our results suggest that this nationwide hepatitis A outbreak was associated with eating 1 batch of semidried tomatoes imported from Turkey and processed in France. Infected food handlers are the most frequently documented source of contamination by HAV of food items, but food also can be contaminated by contact of products or machinery with contaminated water (*2*). Therefore, the tomatoes may have been contaminated during processing by the supplier in France, during production in Turkey, or during growing. Fecal contamination of foods that are not subsequently cooked is a potential source of HAV, and the virus remains

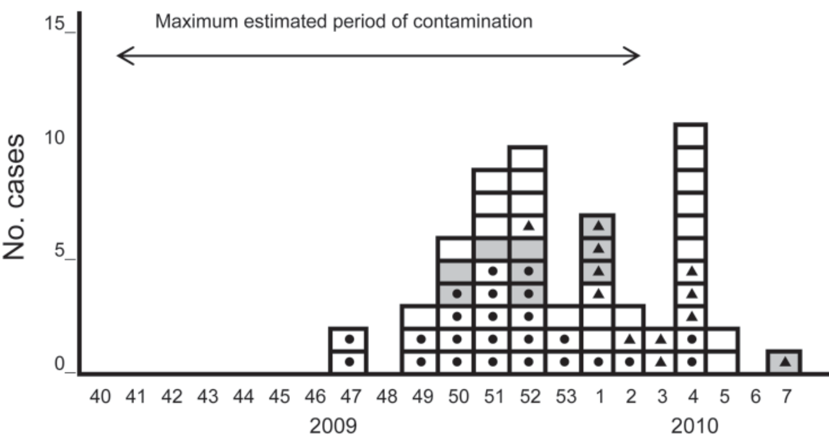

Figure. Weekly distribution of hepatitis A cases, by date of onset, France, November 2009–February 2010. White boxes indicate confirmed cases; gray boxes indicate probable cases; dots indicate patient consumed semidried tomatoes; triangles indicate secondary cases. French calendar designates 53 weeks in 2009 (week 53, December 28, 2009–January 3, 2010).

infectious for long periods, even after freezing (*3*). Various fresh or frozen produce have been associated with hepatitis A outbreaks (*4,5*).

Recently, 3 other hepatitis A outbreaks were associated with eating semidried tomatoes: in Australia in May and November 2009 and in the Netherlands in 2010 (*6–9*). All 4 outbreaks were caused by highly similar IB strains, although the French outbreak strain differed by 2 nt from the Australian strain (based on a 300-nt fragment of the VP1–2A part of the genome), and by 3 nt from the Dutch strain (on the basis of a 430-nt fragment) (*8*).

During the past decade, hepatitis A incidence has decreased considerably in western Europe (*10*). The low incidence and low vaccine coverage have led to a high proportion of susceptible persons, which creates the potential for extended hepatitis A outbreaks if contaminated products are widely distributed. Imported products from regions with high endemicity are widely distributed throughout Europe, and some have a long shelf life, (especially if frozen). Semidried tomatoes should be considered a potential vehicle of transmission in foodborne outbreaks of HAV.

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Tetracycline-Resistant *Vibrio cholerae* O1, Kolkata, India

To the Editor: Cholera, caused by toxigenic strains of *Vibrio cholerae* O1 or O139, continues to be a major cause of illness and death, particularly in developing countries. Treatment consists of early administration of rehydration therapy with appropriate oral or intravenous fluids. The World Health Organization recommends antimicrobial drug treatment for severely dehydrated patients with suspected cholera because it substantially shortens the duration of diarrhea by reducing the volume of watery stools, decreases fluid requirements, and limits transmission by decreasing fecal excretion of *V. cholerae* (*1*). The progressive increase in resistance to multiple drugs among strains causing clinical cases of cholera in developing countries is becoming a serious concern. We report the emergence of tetracycline-resistant *V. cholerae* O1 in a well-defined population in Kolkata, India, during 2007–2009.

During a 6-year surveillance period (2004–2009), we conducted a prospective, community-based study at an impoverished urban site in Kolkata. The goals of the study were to estimate the prevalence of cholera, describe its epidemiology, and identify potential risk factors that could be addressed by public health strategies.

Rectal swabs samples from patient with diarrhea were obtained, placed in Cary-Blair transport medium, and transported to the laboratory where they were processed for isolation and identification of *Vibrio* spp. Specimens were plated directly onto thiosulphate citrate bile salt sucrose agar (Eiken Chemical Company, Tokyo, Japan). The specimens were plated directly onto thiosulfate citrate bile salt sucrose agar. They were then incubated in alkaline peptone water (pH 8.6) for 6–8 h at 37°C and then plated onto the agar. After overnight incubation at 37°C, suspected colonies were tested biochemically and confirmed by slide agglutination with polyvalent O1 and monovalent Ogawa and Inaba antiserum (Difco Laboratories, Detroit, MI, USA).

Antimicrobial drug susceptibility testing was performed by using the disk diffusion technique on Mueller-Hinton agar (Difco Laboratories) with commercial disks (Oxoid, Cambridge, UK) and appropriate control strains (*2*). The MIC of tetracycline was determined with 101 randomly selected strains by Etest (AB Biodisk, Solna, Sweden) following manufacturer's instructions.

During the 2004–2009 surveillance period, we isolated 809 *V. cholerae* O1 organisms, among which 624 (77%) were Ogawa and 185 (23%) were Inaba serotypes. The latter became the predominant serotype only in 2006. In 2007, a sudden upsurge in tetracycline resistance was noted among *V. cholerae* isolates, from 1% in 2004 to 76% in 2007 before decreasing to ≈50% in 2009. An increase in resistance to furazolidone and trimethoprim/sulfamethoxazole was also observed during the same period. Of the strains that were resistant to tetracycline, 99% were also resistant to furazolidone and trimethoprim/sulfamethoxazole (online Appendix Figure, www.cdc.gov/EID/content/17/3/568-appF.htm).

Among the tetracycline-resistant isolates (101 randomly selected strains), 43% had high-level resistance (MIC ≥16 µg/mL). In addition, 57% of *V. cholerae* O1 organisms had reduced susceptibility (i.e., MICs ranged from 8 µg/mL to 16 µg/mL).

Tetracycline is the drug of choice for treating cholera (*1*); however, during the 6-year period, we observed the emergence of tetracycline resistance among *V. cholerae* O1 isolates and a sudden upsurge in such resistance in 2007 when 76% of the isolates were resistant. Tetracycline resistance was also reported by Mhalu et al. (*3*), from an epidemic of cholera in Tanzania, where 76% of isolates were found to be resistant after 5 months of extensive use of this drug for treatment and prophylaxis. In a similar situation, the extensive prophylactic use of tetracycline triggered the rapid emergence and spread of tetracycline-resistant strains in Madagascar (*4*). Tetracycline is not used for prophylaxis in Kolkata, a known cholera-endemic area. Nonetheless, the emergence of resistant strains in our study area is not surprising because similar tetracycline-resistant strains of *V. cholerae* have been reported in Bangladesh (*5*), in Mozambique (*6*), and in another study of Kolkata (*7*). Notably, tetracycline-resistant *V. cholerae* O1 strains have also been responsible for major epidemics in Latin America, Tanzania, Bangladesh, and Zaire (*8*).

In our study, resistance to tetracycline among *V. cholerae* O1 isolates was <10% during 2004–2006. The reasons for the sudden rise of resistant strains in 2007 and their continued persistence are still unclear. Detailed molecular studies are underway to find the explanation. Alternative drugs, such as the newer fluoroquinolones, possess excellent activity against *V. cholerae* O1 and O139 serogroups. However, increased resistance to newer fluoroquinolones, such as ciprofloxacin and norfloxacin, among *V. cholerae* strains belonging to O1 serogroup has also been reported (*9*).

Resistance to commonly used antimicrobial drugs represents a critical public health problem because it complicates treatment and may result in longer hospital stays for patients. In addition, most of the population in developing countries cannot afford the newer and more expensive drugs. Our findings emphasize the need for continued

surveillance of antimicrobial drug susceptibility patterns of *V. cholerae*. Providing early information on antimicrobial drug susceptibility to practitioners in affected areas will lessen the illness and death from this devastating disease.

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Neisseria meningitidis Strain of Unknown Serogroup, China

To the Editor: *Neisseria meningitidis* is a major public health hazard in many parts of the world. This organism is classified into 13 serogroups, and most meningococcal disease is caused by strains that express 1 of the 5 types of capsular polysaccharides (A, B, C, Y, and W135). In the natural reservoir of the human nasopharynx, strains of *N. meningitidis* that do not fit into 1 of the 13 serogroups and are presumably unencapsulated are common. By contrast, rare meningococcal diseases are caused by these nonserogroupable strains. In this article, we describe a case of *N. meningitidis* infection caused by a nonserogroupable strain in the People's Republic of China and the genotype characteristics of this strain.

The patient was a 6-month-old boy who was admitted to a local hospital in Beijing in May 2009. The infection started suddenly with high fever (39°C). *N. meningitidis* infection was confirmed on the basis of the clinical signs and results of laboratory examination. Nausea, vomiting, and neck stiffness developed, and the patient lost consciousness. Physical examination showed a positive Kernig sign and negative Brudzinski sign. The patient's cerebrospinal fluid sample was injected into chocolate agar, in which microbial growth was observed after 24 hours. The API NH system (bioMérieux, Marcy-Etoile, France) showed that the isolate was *N. meningitidis*. However, this strain could not be placed in a serogroup, even after specific antiserum (Remel, Lenexa, KS, USA) was used. No other disease with complement deficiency was detected in the patient. The patient's infection was treated with antimicrobial drugs, and he recovered completely.

We investigated this nonserogroupable *N. meningitidis* strain by multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and subtyping of the variable regions of the genes (*por*A, *por*B, and *fet*A) encoding the outer membrane proteins. MLST indicated that the *fum*C was a new allele with a new number 482. The allele numbers for *abc*Z, *adk, fum*C, *gdh, pdh*C, and *pgm* were 222, 3, 58, 386, 18, and 77, respectively. This strain was assigned a new sequence type number, ST7962.

Among the 44 complexes designed in the MLST database, ST7962 was most similar to the ST4821 complex with 3 identical loci. The PFGE pattern of this strain was compared with the PFGE

patterns in the reference database of *N. meningitidis* from China by using BioNumerics version 5.10 software (Applied Maths, Kortrijk, Belgium). At the time of comparison, the database contained 618 isolates of *N. meningitidis* and 243 PFGE patterns. This strain had a single pattern that was clustered together with the ST4821 complex strains in the cluster tree based on the PFGE patterns. The PorA genotype of the strain was determined to be P1.7–2, 14, which was a genotype associated with the ST4821 complex serogroup C strains that caused outbreaks in China in 2003 (*1*). The *por*B and *fet*A alleles of this strain were 3–18 and F4–21, respectively.

The genetic basis for the reason that this strain was nonserogroupable was studied by PCR and sequencing. PCR showed that this strain had intact capsule genetic islands of *ctr*A-D, *syn*A-C, *lip*A, and *lip*B, and contained *syn*D, encoding the serogroup B polysialyltransferase. The capsular gene clusters were sequenced entirely, and a missense mutation within *syn*D was identified (Figure). The mechanism underlying the capsule phase variation of *N. meningitidis* serogroup B involves a variation caused by slipped-strand mispairing in the polyC tract at the 5′ end of *syn*D (*2*–*4*). A tract of 7 C residues encodes capsular expression, and an insertion or deletion of 1 C results in a missense mutation within *syn*D, thereby leading to nonexpression of the capsule. Nucleotide sequencing of *syn*D of our isolate revealed an insertion of 1 C within the polyC tract. Thus, slipped-strand mispairing within *syn*D was predicted to be the mechanism underlying the nonserogroupability of this strain.

Few reports have described invasive meningococcal disease caused by nonserogroupable *N. meningitidis* strains; the lack of such reports suggests that complement deficiency might be a predisposing factor, and all the reported isolates were determined to be capsule null locus (cnl) strains, which lacked the genetic islands encoding the entire capsule (*5*–*7*). However, the patient described here was not found to have a complement deficiency, and the disease-associated nonserogroupable *N. meningitidis* strain in this study contained the genetic locus of an intact capsule.

In China, meningococcal polysaccharide vaccines A and C have been used for routine immunization. In many countries in Africa, repeated vaccination against *N. meningitidis* serogroups A and C have likely led to a selective increase in the incidence of meningococci of other serogroups, thereby resulting in a changed profile of meningococcal disease (*8*). In recent years, invasive disease caused by *N. meningitidis* serogroup W135 and serogroup X strains has emerged in China (*9*,*10*). Therefore, meningococcal disease caused by serogroups other than A and C as well as nonserogroupable *N. meningitidis* strains appears to be an emerging problem and should be investigated epidemiologically.

Strain	*syn*D	Capsule
MC58 (wild-type)	atg ... tat ctt acc ccc cca cgt aac aat ... taa ... Y L T P P R N N ...	On
100924 (missense mutation)	Insertion of 1 C atg ... tat ctt acc ccc ccc acg taa ... Y L T P P T . stop	Off

Figure. Genetic basis for the *Neisseria meningitidis* strain that cannot be placed in a known serogroup. A predicted slipped-strand mispairing occurred within *synD*, which encodes the serogroup B sialyltransferase. In wild-type *N. meningitidis* serogroup B (MC58), the *syn*D polyC tract contains 7 C residues, and capsule is expressed. When an insertion (as in isolate 100924) of 1 C residue occurs, a result of local denaturation and mispairing followed by replication or repair, a premature stop codon is generated, and the capsule is not expressed.

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etymologia

Pseudoterranova azarasi

[sü-dō-′ter-ə-nō-və a-zär-a-sē]

From the Greek for false, Latin for earth and new, and Japanese for sea lion. First identified in 1878 as a parasite in pinnipeds by Danish scientist Harald Krabbe, who suggested the name *Ascaris decipiens*, the taxonomic designation for these nematodes changed as knowledge of the life cycles and morphologic features of members of the order Ascaridida expanded. In 1998, molecular examination found *Pseudoterranova decipiens*, long thought to be a monotype, consisted of genetically distinct sibling species. Mattiucci et al. proposed *Pseudoterranova azarasi* for 1 of the 5 sibling species, incorporating part of the name *Porrocaecum azarasi*, previously considered a synonym for *Pseudoterranova decipiens*.

Sources: Berland B. *Anisakis* spp. In: Akuffo H, Linder E, Ljungström I, Wahlgren M, editors. Parasites of the colder climates. London: Taylor & Francis; 2003. p. 160–8; Krabbe H. On the ascarides of the seals and toothed whales. Annals and Magazine of Natural History. 1878; 5-S, 2(11):430–2; Mattiucci S, Paggi L, Nascetti G, Ishikura H, Kikuchi K, Sato N, et al. Allozyme and morphological identification of *Anisakis, Contracaecum* and *Pseudoterranova* from Japanese waters (Nematoda, Ascaridoidea). Syst Parasitol. 1998;40:81–92. DOI: 10.1023/A:1005914926720

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Transplant Infections, Third Edition

Raleigh A. Bowden, Per Ljungman, and David R. Snydman, editors

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Transplant infectious diseases, a rapidly advancing field, has developed in the past 2 decades into a well-defined subspecialty of infectious diseases. Because of the specialty's interdisciplinary nature, advances in the basic science of immune tolerance, immunomodulatory therapy, surgical techniques, antimicrobial therapy, pathogen detection, and global surveillance for emerging infections all intersect in this dynamic field. The third edition of the Transplant Infections textbook is, therefore, a welcome update to the 2003 edition and provides an excellent overview of this rapidly changing field.

The contributors to this textbook are primarily infectious disease specialists from many of the largest academic transplant centers worldwide. This textbook's organization reflects its clinical orientation. It begins with an overview of solid organ transplantation and hematopoietic stem cell transplantation; the ensuing chapters review the pharmacology and immunology related to transplant infectious diseases and include useful tables detailing mechanism of action, side effects, and drug interactions. New chapters on infections after solid organ transplantation, donor-derived infections, and infections in developing countries highlight the increasing awareness of these topics. The practical organization of the subsequent chapters into infections by type of transplantation, sites of infection, and class of pathogen remains unchanged from the previous edition and provides a quick reference for clinicians. Despite the new emphasis on donor-derived infections and geographic medicine, the pathogen-specific chapters variably cover these angles. New chapters on emerging and rare viral infections and on travel medicine, vaccines, and transplant tourism provide brief overviews of these complex topics. A more thorough review of the impact of emerging infections and geographic medicine on transplant medicine would have been useful, given the current era of unprecedented population mobility and the corresponding risk of exposure to emerging pathogens.

The shortcoming of textbooks about rapidly changing fields is that the details may become quickly outdated. For example, the chapter on infection control issues after hematopoietic stem cell transplantation unfortunately does not include reference to the recently updated 2009 Guidelines for Preventing Infectious Complications among Hematopietic Cell Transplantation Recipients, although it refers to the Centers for Disease Control and Prevention website for updated information.

This textbook excels as an orientation and update of the field of transplant infectious diseases for the practicing clinician; infectious diseases specialists, surgeons, researchers, and public health practitioners will benefit from its practical organization and style. For those seeking more comprehensive detail and updated information, it may fall slightly short, but it provides essential background for further exploration.

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Edvard Munch (1863–1944) *The Sick Child* (1907) Oil on canvas. Dimensions: support: 1,187 mm × 1,210 mm frame: 1,371 mm × 1,393 mm × 108 mm painting. The Munch Museum/The Munch-Ellingsen Group/Artist Rights Society, NY. Image copyright, Tate, London, 2011

From My Rotting Body, Flowers Shall Grow, and I Am in Them, and That Is Eternity

—Edvard Munch

Polyxeni Potter

"My afflictions belong to me and my art—they have become one with me. Without illness and anxiety, I would have been a rudderless ship," wrote Edvard Munch, acknowledging that pain and suffering fueled his creativity and guided his art. In an era gripped by world wars and a deadly flu pandemic and shaken by Sigmund Freud and Charles Darwin, he sought to recreate his troubled childhood and his lifelong struggle with physical and mental illness. "My art is really a voluntary confession and an attempt to explain to myself my relationship with life—it is, therefore, actually a sort of egoism, but I am constantly hoping that through this I can help others achieve clarity."

Childhood trauma started at age 5, when his mother died of tuberculosis, and continued at 13 with loss of his beloved sister Sophie to the same disease. The deaths unsettled this Norwegian household already compromised by poverty. The family lived in tenement housing plagued by tuberculosis and bronchitis—scourges of the working class. The father, an underpaid medical officer, was "temperamentally nervous and obsessively religious—to the point of psychoneurosis." Another sister had to be hospitalized for mental illness. A brother died. "Often I awoke in the middle of the night, gazing around the room in wild fear—was I in hell?" Munch had childhood bouts of respiratory infections, pneumonia, tuberculosis, and rheumatic fever. "I inherited two of mankind's most frightful enemies—consumption and insanity."

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After a stint as engineering student to appease his father, he enrolled in art school to study painting, "an unholy trade." He fell in with a bohemian crowd, absorbing their philosophy for a lifetime; came under the influence of anarchist and nihilist Hans Jaeger, who admonished him to "write his life"; and enrolled in the Royal School of Art and Design of Christiania (now Oslo) to study under naturalist painter Christian Krohg. His early work, which experimented with various styles, was labeled "unfinished" and was viewed with universal hostility as "impressionism carried to the extreme."

Munch did not reject any of the styles he studied at home or later in Paris, where he worked under Léon Bonnat. He used naturalism, impressionism, and linear concepts such as those in the work of Henri de Toulouse-Lautrec and Art Nouveau practitioners. Like his friends Henrik Ibsen and August Strindberg, he had a flair for drama, which he

developed fully in sets with characters inside his paintings. Like the symbolists, with whom he is often identified, he used metaphorical elements to create emotional scenes and avoided perspective, chiaroscuro, and naturalistic color. Instead he used stylized or exaggerated objects, moods, and thoughts. In his mature works, canvas and painted surface became a means of expression and he the forerunner of a new modernist movement, expressionism.

Increasingly concerned with the expressive representation of emotions, he addressed the fundamental elements of human experience—birth, love, death—over and over again, exploring them throughout his life, which was filled with unsatisfactory relationships, a hostile public, continued illness, breakdowns and hospitalizations, and a bout in 1918 of the Spanish Flu. These he transformed into art filled with melancholy. He died of pneumonia at 80.

Munch considered *The Sick Child*, on this month's cover, his first "soul painting," a break from impressionism. He painted the subject six times and also explored it in lithography, drypoint, and etching. The painting was not well received because of its "roughness" and "sketchiness." But Christian Krohg liked it: "He sees only the essential, and that, naturally, is all he paints. For this reason Munch's pictures are as a rule 'not complete.'"

"As for *The Sick Child*," Munch wrote, "it was the period I think of as the age of the pillow." Many artists did pictures of children on their pillows. "What I wanted to bring out—is that which cannot be measured—I wanted to bring out the tired movement in the eyelids—the lips must look as though they are whispering—she must look as though she is breathing—I want life—what is alive." Since hailed as the first expressionist masterpiece, the painting shows his sister Sophie, on her deathbed, turned toward a dejected figure nearby. The composition is cramped and isolating, patient and mourner trapped in their predicament.

The emotional effect comes not from a naturalistic physical presentation but from somewhere within the figures. The light emanates from the pillow and from the face of the girl, which seems transparent, a fever-induced vision already in the world of spirits. The mask-like features and color patchiness, the lack of facial expression betray the nature of the child's illness. The woman at the bedside, head bowed against the contracted chest, hands resting on the blanket, grieves inwardly. This non-violent display of emotion resonates, as if the nervous excitement not discharged in muscular action fuels associations of melancholy to arouse increased feeling. The painting is the artist's recall of his own response to the death scene. "I do not paint what I see. I paint what I have seen."

The paint is thinned and scored for intensity, the strong surrounding colors contrasting with the faint face of the child. Red on the furniture, the child's hair, and repetitively in the background invokes blood, harbinger and dominant presence of tuberculosis in the scene.

The Sick Child portrays a dying adolescent, her physical and spiritual attractiveness heightened, as was believed, by her very illness. The pathos of youth caught between life and death recalls the words of John Keats (1795–1821), whose own life and immense promise were cut short by tuberculosis. The poet embraced the opposites in life: love and loss, pleasure and pain, the "inextricable contrarieties of life," part of the human condition. Therefore, beauty, joy, and life are valuable because they are transitory and eventually become their very opposites. And so does Melancholy, "She dwells with Beauty—Beauty that must die; / And Joy, whose hand is ever at his lips / Bidding adieu; and aching Pleasure nigh, / Turning to Poison while the bee-mouth sips."

Consumptive drama has subsided in our times, with tuberculosis viewed less as romantic tragedy or isolating contagion and more as serious but treatable disease. Sadly still a global threat, particularly among AIDS patients and the underprivileged, tuberculosis remains as debilitating and traumatizing as in Munch's experience and art, even though the tubercle bacillus as its etiologic agent was discovered during his lifetime.

The relationship between illness and creativity, embraced as pivotal by Munch though a long-standing academic discussion, has run the gamut from prophetic to tiresome. But Keats' notion of "inextricable contrarieties" still works. The genius of human invention exemplified by Robert Koch (1843–1910) and so many others inevitably meets its direct opposite, logistical failure or failure of will. Thus such brilliant discoveries as knowing what causes tuberculosis, how it is spread, and how to treat it have not yet eliminated this disease. "Aye, in the very temple of Delight / Veiled Melancholy has her sov'reign shrine."

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Upcoming Issue

Bacterial Phenotype Variants in Group B Streptococcal Toxic Shock Syndrome

Legionella longbeachae and Legionellosis

Hand Hygiene Campaigns, Influenza, and School Absenteeism, Cairo, Egypt

Mumps and Vaccination, England and Wales

Bordetella petrii Infection with Long-lasting Persistence

Geographic Distribution of *Coxiella burnetii* Genotypes in Ruminants during Q Fever Outbreak, the Netherlands

Orthopoxvirus in Eurasian Lynx, Sweden

Diarrheagenic Pathogens in Mixed Infections

Pneumococcal Carriage after Vaccination Program

Shedding of Pandemic (H1N1) 2009 Virus among Health Care Personnel, Seattle, Washington

Highly Virulent Isolate of African Swine Fever Virus

Nosocomial Pandemic (H1N1) 2009, United Kingdom, 2009–2010

Should Remaining Stockpiles of Smallpox Virus (Variola) Be Destroyed?

Remaining Questions about Clinical Variola Major

High Rates of USA400 *Staphyolcoccus aureus* Infection, Northern Canada

Vaccinia Virus Infections and Martial Arts Gym, Maryland, 2008

Hepatitis A Virus Vaccine Escape Mutants and New Serotype Emergence

Highly Pathogenic Avian Influenza Virus Infection in Feral Raccoons, Japan

Seasonality of Cat-Scratch Disease, France

Transmission of Vaccinia Virus Following Military Vaccination, 2010

Human Metapneumovirus Infection in Wild Mountain Gorillas

Complete list of articles in the April issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

April 1–4, 2011
Annual Scientific Meeting of The Society for Healthcare Epidemiology of America
(SHEA) 2011
Dallas, Texas, USA
http://www.shea2011.com

April 13–16, 2011
The Denver TB Course
Denver, CO, USA
http://www.njhealth.org/TBCourse

July 8–10, 2011
International Society for Infectious Diseases Neglected Tropical Diseases Meeting (ISID-NTD)
Boston, MA, USA
http://ntd.isid.org

October 12–15, 2011
The Denver TB Course
Denver, CO, USA
http://www.njhealth.org/TBCourse

November 16–19, 2011
7th World Congress of the World Society for Pediatric Infectious Diseases (WSPID 2011)
Melbourne, Australia
http://www.kenes.com/wspid2011/mailshot/ms3.htm

2012
June 13–16, 2012
15th International Congress on Infectious Diseases (ICID)
Bangkok, Thailand
http://www.isid.org/15th_icid

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Article Title

Staphylococcus aureus Infections in US Veterans, Maryland, USA, 1999–2008

CME Questions

1. On the basis of the current retrospective cohort study by Dr. Tracy and colleagues, which of the following statements about the overall incidence of *Staphylococcus aureus* infections in the Veterans Affairs Maryland Healthcare System between fiscal years 1999 and 2008 is most likely correct?

A. Incidence of all *S. aureus* infections significantly decreased
B. Changes in incidence of all *S. aureus* infections were most striking during and following 2005
C. 10-year average overall incidence per 100,000 veterans was 749 ± 132
D. The findings are applicable to pediatric units

2. You are an infection-control officer in a Maryland veterans hospital. On the basis of the study by Dr. Tracy and colleagues, which of the following statements about trends in invasive vs noninvasive *S. aureus* infections, methicillin susceptibility, and location of onset and infection site is most likely to apply to infection control at your hospital?

A. About half of *S. aureus* infections are likely to be invasive
B. A projected increase in overall *S. aureus* infections is most likely to be driven by invasive, methicillin-sensitive, hospital-onset infections
C. More than half of all noninvasive infections are likely to be skin and soft tissue infections
D. Hospital-onset invasive MRSA infections are likely to significantly increase

3. As the infection-control officer described in question 2, which of the following hospital infection-control practices do you think would be most likely to reduce incidence of invasive *S. aureus* infections, as based on their use in the VA hospital studied by Dr. Tracy and colleagues?

A. Hand-washing with soap and water rather than using alcohol-based hand gels
B. Central-line bundles
C. Surveillance cultures for methicillin-sensitive *S. aureus*
D. Soap-and-water rather than chlorhexidine bathing for surgical patients

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Article Title

Tuberculosis among Health Care Workers

CME Questions

1. Based on the above meta-analysis by Dr. Baussano and colleagues, which of the following statements about the risk for healthcare workers (HCWs) of developing incident latent tuberculosis infection (LTBI) is most likely correct?

A. There is no clear evidence that TB is an occupational disease

B. Annual risk for LTBI among HCWs is not associated with background TB incidence in the population

C. Stratified pooled estimates for the LTBI rate for the countries with an estimated low (<50 cases/100,000) TB incidence was 1.8%

D. Stratified pooled estimates for the LTBI rate for the countries with an estimated high (>100 cases/100,000) TB incidence was 8.4%

2. You are asked by the World Health Organization to consult on expected risk of developing TB disease for HCWs worldwide. Based on the above meta-analysis, which of the following statements would be most likely to appear in the report of your findings?

A. The background TB incidence in the population is not associated with incidence rate ratios (IRRs) for TB disease

B. Median estimated population attributable fraction (PAF) percentage for TB disease exceeds 2%

C. For TB disease, IRR estimate is 2.4 for countries with estimated low or intermediate TB incidence, and 3.7 for countries with estimated high TB incidence

D. The impact of TB in HCWs on global TB burden exceeds 10%

3. As the consultant described in Question 2, which of the following statements about suggested TB infection control measures is most likely to appear in your report, based on the above meta-analysis?

A. Infection transmission control strategies are not likely to be effective

B. Implementing TB infection control measures is recommended only at select healthcare facilities caring for patients suspected of having infectious TB

C. Administrative controls alone are ineffective in reducing the risk for TB among HCWs

D. Managerial activities, administrative controls, N95 respirators, and engineering controls are all potentially effective in reducing the risk for TB among HCWs

Activity Evaluation

1. The activity supported the learning objectives.

Strongly Disagree				Strongly Agree
1	2	3	4	5

2. The material was organized clearly for learning to occur.

Strongly Disagree				Strongly Agree
1	2	3	4	5

3. The content learned from this activity will impact my practice.

Strongly Disagree				Strongly Agree
1	2	3	4	5

4. The activity was presented objectively and free of commercial bias.

Strongly Disagree				Strongly Agree
1	2	3	4	5

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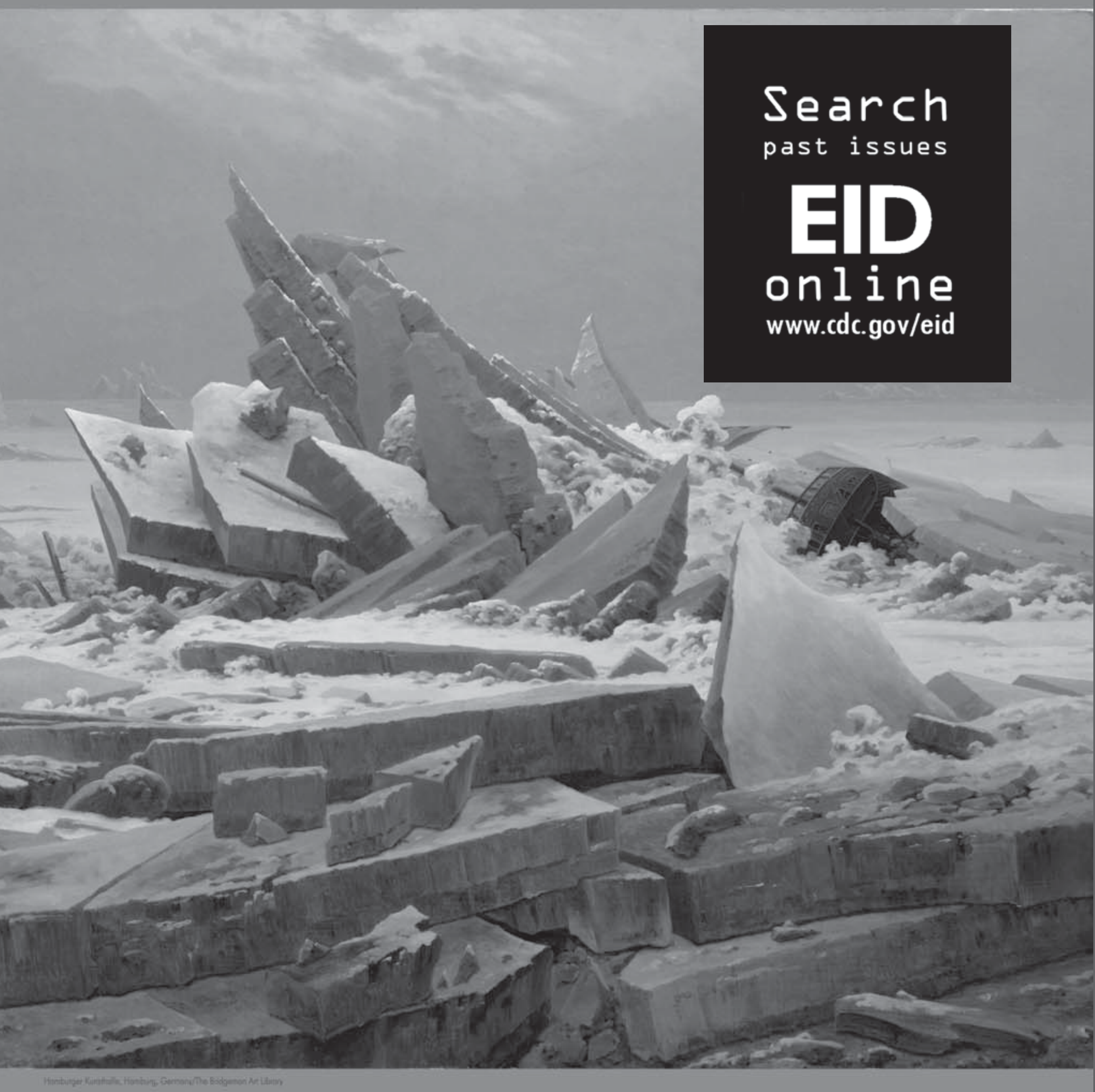

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